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RAMP1 and RAMP3 Differentially Control Amylin's Effects on Food Intake, Glucose and Energy Balance in Male and Female Mice

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RAMP1 und RAMP3 kontrollieren die Effekte von Amylin auf Futteraufnahme, Glukose und Energiehaushalt in männlichen und weiblichen Mäusen

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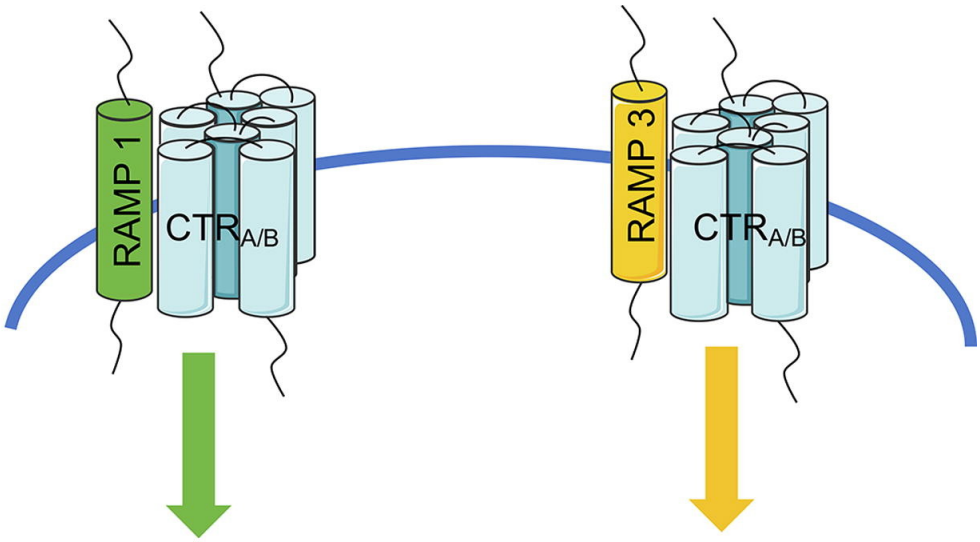
Amylin ist ein Peptid aus dem endokrinen Pankreas und nimmt eine Schlüsselrolle in der Kontrolle von Futteraufnahme und Energiehaushalt ein, wobei es mehrheitlich an drei Rezeptoren bindet (AMY 1-3). AMY 1-3 bestehen aus einem Calcitonin-Rezeptor (CTR) und jeweils einem rezeptor-aktivität-modifizierenden Protein (RAMP1-3). In dieser Studie verwenden wir RAMP1, RAMP3 und RAMP1/3 globale knockout (KO) Mausmodelle um herauszufinden, wie sich das Fehlen einzelner oder beider Komponenten auf Futteraufnahme, Glukosehaushalt und Metabolismus auswirken. Von allen Mausmodellen mit fehlenden RAMPs hatten nur die RAMP1/3-KO, die eine Diät mit erhöhtem Fettgehalt (HFD) erhalten hatten, ein erhöhtes Körpergewicht. Männliche Mäuse mit RAMP3 KO auf normaler Diät und RAMP1/3 KO auf HFD hatten eine reduzierte Glukosetoleranz. Alle Mausmodelle wurden dann mit Amylin und Lachs-Calcitonin (sCT), ein Agonist für den Amylin-Rezeptor, behandelt, um deren Auswirkung auf die Futteraufnahme zu vergleichen. RAMP1/3 KO waren nicht sensitiv auf beide Behandlungen, während RAMP3 KO nur auf sCT, und RAMP1 KO nur auf Amylin reagierten. Generell wogen weibliche Mäuse weniger als männliche, aber nur bei RAMP1 KO konnte man eine klare Trennung zwischen Geschlechtern bezüglich Futteraufnahme beobachten. Letzten Endes zeigt diese Studie, dass der RAMP1-Subtyp hauptsächlich am Fetthaushalt beteiligt ist, während der RAMP3-Subtyp für Glukosehauhalt und den anorektischen Effekt von Amylin verantwortlich ist.



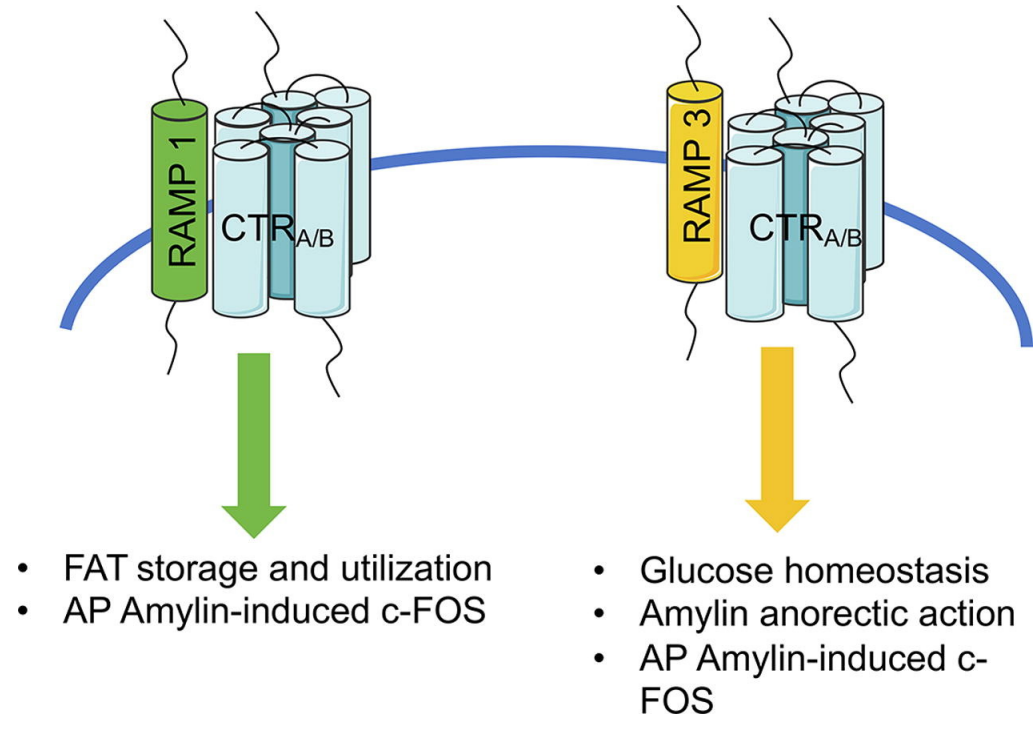
- Fettspeicherung und -verwertung
- Amylin-induziertes c-Fos in AP

- Glukosehaushalt
- Anorektischer Effekt von Amylin
- Amylin-induziertes c-Fos in AP

RAMP1 and RAMP3 Differentially Control Amylin's Effects on Food Intake, Glucose and Energy Balance in Male and Female Mice

Bernd Coester, Sydney W Pence, Soraya Arrigoni, Christina N Boyle, Christelle Le Foll, Thomas A Lutz

Amylin is a pancreatic peptide, which acts as a key controller of food intake and energy balance and predominately binds to three receptors (AMY 1–3). AMY 1–3 are composed of a calcitonin core receptor (CTR) and associated receptor-activity modifying proteins (RAMPs) 1–3. Using RAMP1, RAMP3 and RAMP1/3 global KO mice, this study aimed to determine whether the absence of one or two RAMP subunits affects food intake, glucose homeostasis and metabolism. Of all the RAMP-deficient mice, only high-fat diet fed RAMP1/3 KO mice had increased body weight. Chow-fed RAMP3 KO and high-fat diet fed 1/3 KO male mice were glucose intolerant. Fat depots were increased in RAMP1 KO male mice. No difference in energy expenditure was observed but the respiratory exchange ratio (RER) was elevated in RAMP1/3 KO. WT and RAMP1, RAMP3, and RAMP1/3 KO male and female littermates were then assessed for their food intake response to an acute intraperitoneal injection of amylin or its receptor agonist, salmon calcitonin (sCT). RAMP1/3 KO were insensitive to both, while RAMP3 KO were responsive to sCT only and RAMP1 KO to amylin only. While female mice generally weighed less than male mice, only RAMP1 KO showed a clear sex difference in meal pattern and food intake tests. Ultimately, the results from this study provide evidence for a role of RAMP1 in mediation of fat utilization and a role for RAMP3 in glucose homeostasis and amylin's anorectic effect.



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RAMP1 and RAMP3 Differentially Control Amylin's Effects on Food Intake, Glucose and Energy Balance in Male and Female Mice

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Abstract—Amylin is a pancreatic peptide, which acts as a key controller of food intake and energy balance and predominately binds to three receptors (AMY 1–3). AMY 1–3 are composed of a calcitonin core receptor (CTR) and associated receptor-activity modifying proteins (RAMPs) 1–3. Using RAMP1, RAMP3 and RAMP1/3 global KO mice, this study aimed to determine whether the absence of one or two RAMP subunits affects food intake, glucose homeostasis and metabolism. Of all the RAMP-deficient mice, only high-fat diet fed RAMP1/3 KO mice had increased body weight. Chow-fed RAMP3 KO and high-fat diet fed 1/3 KO male mice were glucose intolerant. Fat depots were increased in RAMP1 KO male mice. No difference in energy expenditure was observed but the respiratory exchange ratio (RER) was elevated in RAMP1/3 KO. RAMP1 and 1/3 KO male mice displayed an increase in intermeal interval (IMI) and meal duration, whereas IMI was decreased in RAMP3 KO male and female mice. WT and RAMP1, RAMP3, and RAMP1/3 KO male and female littermates were then assessed for their food intake response to an acute intraperitoneal injection of amylin or its receptor agonist, salmon calcitonin (sCT). RAMP1/3 KO were insensitive to both, while RAMP3 KO were responsive to sCT only and RAMP1 KO to amylin only. While female mice generally weighed less than male mice, only RAMP1 KO showed a clear sex difference in meal pattern and food intake tests. Lastly, a decrease in CTR fibers did not consistently correlate with a decrease in amylin- induced c-Fos expression in the area postrema (AP). Ultimately, the results from this study provide evidence for a role of RAMP1 in mediation of fat utilization and a role for RAMP3 in glucose homeostasis and amylin's anorectic effect.

This article is part of a Special Issue entitled SI: Neuroscience of Obesity.

Key words: amylin, RAMP, area postrema, glucose homeostasis, metabolism, fat.

INTRODUCTION

Amylin is synthesized by pancreatic β-cells and is co-released with insulin in response to food intake and increasing glucose levels (Ogawa et al., 1990). The amylin receptor (AMY) is composed of a core calcitonin receptor (CTR) a or b (Hay et al., 2005; Lutz, 2012), a class B1 G-protein-coupled receptor (GPCR), which heterodimerizes with one or several receptor activity-modifying proteins (RAMPs 1, 2, 3) (McLatchie et al., 1998; Qi et al., 2008) to form AMY1-3. These components are expressed in the area postrema (AP), nucleus of the solitary tract (NTS), the lateral hypothalamic area, ventromedial (VMN) and arcuate (ARC) hypothalamic nuclei, and the ventral tegmental area (Hilton et al., 1995; Le Foll et al., 2015; Mietlicki-Baase et al., 2013). In the AP, we have found that most individual CTRa-positive neurons co-express one or more RAMP subunit. In addition, the same study showed that exogenous amylin acutely down-regulated RAMP1 and RAMP3 mRNA levels, but not CTR, suggesting a possible negative feedback mechanism of amylin on components of its own receptor (Liberini et al., 2016). Several studies have demonstrated that the AP is the primary site for peripheral amylin's satiating effects (Lutz et al., 2001; Riediger et al., 2001; Riediger et al., 2004), but the ventromedial hypothalamus (VMH = ARC + VMN) is also a direct target for amylin signaling. We have shown that amylin can activate ERK signaling in proopiomelanocortin (POMC) neurons of the ARC, independent of an action in the AP, and that this effect is blunted in RAMP1/3 KO mice (Lutz et al., 2018). Moreover, amylin can synergize with leptin in the

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Abbreviations: ARC, arcuate nucleus of the hypothalamus; CTR, calcitonin core receptor; EE, energy expenditure; FM, fat mass; GPCR, G-protein-coupled receptor; IMI, intermeal interval; LBM, lean body mass; POMC, proopiomelanocortin; RAMPs, receptor-activity modifying proteins; RER, respiratory exchange ratio; sCT, salmon calcitonin; VMN, ventromedial nucleus of the hypothalamus.

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VMN by enhancing leptin-induced p-STAT3 signaling (Le Foll et al., 2015; Turek et al., 2010). Amylin signaling is also essential for the normal development of axonal outgrowth from POMC and agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons to the PVN during the neonatal period; RAMP1/3 KO mice have significantly decreased α-melanocyte-stimulating hormone (MSH) and AgRP axonal fiber outgrowth from ARC to PVN (Lutz et al., 2018). While amylin's effect on POMC neurons is direct, its effect on AgRP/NPY neurons seems to be indirectly mediated by interleukin-6 (IL-6) (Larsen et al., 2016; Le Foll et al., 2015).

In addition to amylin, RAMPs can bind other peptides when combined with other GPCR (Barbash et al., 2017). When associated to the calcitonin receptor-like receptor (CLR), RAMPs form the adrenomedullin (AM) receptors and can bind calcitonin-gene related peptide (CGRP) and adrenomedullin (Hay et al., 2015; Hay et al., 2017). When RAMP1 is combined to CLR it preferentially binds to CGRP, while RAMP3/CLR binds AM and AM2. CGRP and AM are known to be potent vasodilators (Hendrikse et al., 2019). CGRP can also act centrally to modulate neuronal activity and stimulation of CGRP-expressing neurons in the lateral parabrachial nucleus reduces food intake (Campos et al., 2016; Essner et al., 2017). Further, CGRP is also able to interact with AMY1 (Simms et al., 2018). Thus, the association of RAMPs with these GPCR modulates signaling of several hormonal systems.

To determine the contribution of individual RAMPs to whole-body energy balance and glucose homeostasis, male and female mice globally deficient in RAMP1, RAMP3 or both RAMP1 and 3 were metabolically phenotyped. We next determined the specific role of RAMP1 and RAMP3 in regulating amylin's control of food intake and activation of AP neurons. Given the fact that amylin enhances leptin signaling in the VMH, leptin's action on food intake was also tested in these KO mice. Experiments were performed in both male and female KO mice, which enabled the analysis of sex as a factor influencing RAMP-deficient phenotypes.

EXPERIMENTAL PROCEDURES

Animal husbandry and diet

All animals were maintained in a temperature-controlled (21 ± 2 °C) room on a 12:12 h light/dark schedule with lights off at 1000 h. Food and water were provided ad libitum. Unless otherwise specified, the animals were fed a standard chow diet (Diet 3436, Provimi Kliba AG, Kaiseraugst, Switzerland; energy content: 3.15 kcal/g, 65.4% energy from carbohydrates, 12.3% from fat and 22.4% from protein as percent of total energy content). RAMP1/3 double KO mice (background: 129S6/SvEv; kindly donated by Kathleen Caron, Univ. North Carolina, Chapel Hill, NC, USA) (Dackor et al., 2007) were bred in our facility. WT mice were age matched and derived from the RAMP3 +/− colony. RAMP3 +/− and RAMP1 +/− mice (background: 129S6/SvEv; kindly donated by Kathleen Caron) (Dackor et al., 2007) were also bred in our facility and WT and KO littermates were used for our experiments. Animals were genotyped using the following primers: RAMP1 forward TCATGGGGACCTTTAGGTAAGC, RAMP1 reverse ACAGCAATCCTTCTACCTCAACAC, RAMP3 WT band is detected using R3-1: GTGCTCAAGGGTTCTGTCTG and R3-10: GACCTGGTTCATCTCTGGCTCC and RAMP3 null band is detected using R3-10: GACCTGGTTCATCTCTGGCTCC and neo-60: GCTTCCTCTTGCAAAACCACA (Dackor et al., 2007). The animals were kept in an enriched environment with cardboard houses, wood chip bedding, and nesting material. Mice were maintained in group-housing conditions unless specified. All procedures were approved by the Veterinary Office of the Canton Zurich, Switzerland.

RAMP1/3 KO mice experiments (Overview of the experimental groups depicted in Fig. 7)

Cohort 1: Group-housed 4 wk-old male WT (n = 8) and male RAMP1/3 KO (n = 8) mice were fed chow diet for 4 wks. They were then single-housed and fed 45% high fat diet (energy content: 4.71 kcal/g, 35% energy from carbohydrates (17% sucrose), 45% from fat, 20% from protein as percent of total energy content; D12451 Research Diet) for 7 wks. Weekly body weight and food intake were measured starting 1 h before dark onset. After each diet period, mice underwent an oral glucose tolerance test (i.e at 8 and 15 wk-old). Mice were then transferred to the indirect calorimetry cages (Phenomaster TSE Systems, Bad Homburg, Germany) and their energy expenditure and respiratory exchange ratio were assessed; the animals were maintained on 45% HF diet for this period. Terminally, 17 wk-old mice were euthanized with an overdose of pentobarbital (100 mg/kg i.p.) and their body composition was analyzed (CT scan, La Theta LCT-100; see below).

Cohort 2: 6 wk-old male and female WT (n = 12) and RAMP1/3 KO (n = 13) mice were fed chow diet and were individually housed in cages equipped with BioDAQ Food Intake Monitors (Research Diet, New Brunswick, NJ, USA) to continuously measure food intake and meal patterns. After one week of habituation and stabilization of the baseline food intake, mice were assessed after a 12 h fast during the light phase for their response to amylin (i.p. 20, 100, 500 μg/kg, H-9475, Bachem, Bubendorf, Switzerland) and salmon calcitonin (sCT, i.p. 10 μg/kg, 4033011.0001, Bachem) in a randomized cross-over manner. Terminally, 12 wk-old mice were injected at dark onset, after a 12 h fast in the light phase, with vehicle or 50 μg/kg of amylin and transcardially perfused (n = 6–7/group).

Cohort 3: 9 wk-old male WT (n = 7) and RAMP1/3 KO (n = 7) male mice were fed chow diet and were individually housed in the BioDAQ-equipped cages. After one week of habituation and stabilization of the baseline food intake, mice were tested after a 12 h fast for their response to leptin (i.p. 5 mg/kg, Peprotech, London, UK) or amylin + leptin (i.p. 50 μg/kg + 5 mg/kg). Mice were then transferred into the indirect calorimetry system and their energy expenditure and respiratory exchange ratio were measured. Terminally, 13 wk-old mice were injected at dark onset, after a 12 h fast, with vehicle or 50 μg/kg of amylin and perfused

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(n = 3–4/group). The body carcasses were kept for body composition measurement.

RAMP3 KO mice experiments (See flowchart in Fig. 7)

Cohort 1: 16 wk-old male and female WT and RAMP3 KO littermate mice were fed chow diet and were individually housed in BioDAQ-equipped cages. (n = 7–11/group). Mice were assessed for their response to amylin (i.p. 20, 100, 500 μg/kg) and sCT (i.p. 10 μg/kg). An OGTT was performed at the end of the food intake study at 20 wks old. Terminally, the animals were injected with vehicle (NaCl 0.9%) or 50 μg/kg of amylin and perfused (n = 8/group).

Cohort 2: 12 wk-old male WT and RAMP3 KO littermate mice were fed chow diet and were individually housed in BioDAQ-equipped cages. (n = 8/group). Similarly, mice were assessed for their response to leptin and amylin + leptin. Mice underwent an OGTT at 16–18 wks old and were then transferred to the indirect calorimetry cages and their energy expenditure and respiratory exchange ratio were assessed. Terminally, mice were euthanized with an overdose of pentobarbital (100 mg/kg) and their body composition was analyzed (n = 8/group).

RAMP1 KO mice experiments (See flowchart in Fig. 7)

Cohort 1: 14 wk-old male and 10 wk-old female WT and RAMP1 KO littermate mice were fed chow diet and were individually housed in the indirect calorimetry system to continuously measure food intake, energy expenditure and respiratory exchange ratio (n = 8/group/sex). After one week of habituation and stabilization of the baseline food intake, mice were assessed for their response to amylin (i.p. 20, 100, 500 μg/kg) and sCT (i.p. 10 μg/kg) similar to the above experiments. An OGTT was performed at the end of the food intake study. Terminally, the animals were injected with vehicle or 50 μg/kg of amylin and perfused (n = 8/group/sex).

Cohort 2: 18 wk-old male and 14 wk-old female WT and RAMP1 KO littermate mice were fed chow diet and were individually housed in indirect calorimetry cages (n = 8/group/sex). Mice were assessed after a 12 h fast for their response to leptin. An OGTT was performed at the end of the food intake study at 22 and 18 wks old respectively. Terminally mice were euthanized with an overdose of pentobarbital (100 mg/kg) and their body composition was analyzed.

Assessment of food intake and meal patterns

Following 7 days of acclimation in the BioDAQ cages, mice were fasted for 12 h during the light phase. At dark onset (10:00 h), food was returned and baseline refeeding was measured for the subsequent 24 h. Meal pattern criteria were an intermeal interval (IMI) of 600 s and a minimal meal size of 0.02 g (Duffy et al., 2018). Meal patterns of the RAMP1 KO mice housed in the indirect calorimetry cages were assessed using similar criteria; however, the meal duration was smaller than for RAMP1/3 and 3 KO mice housed in the BioDAQ cages due to difference in hopper design.

Oral glucose tolerance test

Food was removed 2 h prior to lights off and mice were gavaged at lights off, using an intra-gastric needle (1 mm × 30 mm) with 2 g/kg of D-glucose. Blood was sampled from the tail prior to gavage (0) and at 15, 30, 60, 90, and 120 min post-gavage for glucose measurement (Glucometer: Breeze2, Bayer, Zurich, Switzerland). The area under the curve from 0 min to 120 min was calculated from above baseline value (0 min time-point). Additionally, baseline blood was harvested after a 2 h fast, 2 days before the OGTT, under isoflurane anaesthesia (2%) by puncturing the sublingual vein. Blood was collected in 100 μl EDTA tubes containing protease inhibitors (Sigma Aldrich P2714, Buchs, Switzerland) centrifuged at 10,000 rpm for 10 min and the plasma was stored at −80 °C until further processing.

Blood analysis

Plasma insulin and leptin were measured in duplicates using the mouse metabolic duplex kit (Reference K15124C, Meso Scale Discovery MD, USA) following the manufacturer's instructions. The detection limits are: leptin 43–100,000 pg/mL and insulin 15–50,000 pg/mL.

Indirect calorimetry

A 16-cage TSE PhenoMaster open circuit indirect calorimetry system was used for the determination of O_2 consumption and CO_2 production (TSE Systems; Bad Homburg, Germany). The system was calibrated using precision calibration gases prior to each run. Room air was passed through each cage at a flow rate of 0.41 L/min. Every 20 min, cage air was sampled from each individual cage and analyzed for O_2 and CO_2. From these values, energy expenditure (EE) and respiratory exchange ratio (RER) were calculated based on equations from Weir (Weir, 1949). To account for differences in body weight and body mass composition, EE data were corrected for individual lean body mass (LBM in g) and fat mass (FM in g) using the following equation: LBM + 0.2FM, as recommended by Even and Nadkarni (Even and Nadkarni, 2012; Piattini et al., 2019).

Measurement of body composition

Bodies of the RAMP1/3 KO were CT scanned using a La Theta LCT-100 (Hitachi Aloka Medical Ltd, Steinhausen, Switzerland). The mice were placed supine in the plexiglass holder with an inner diameter of 48 mm. The X-ray source tube voltage was set at 50 kV with 1 mA current. The region between the vertebrae L1–L4 was evaluated for lean and fat mass. LaTheta LCT-100 software automatically distinguishes between visceral and subcutaneous fat; however, each image was examined and corrected if assigned incorrectly.

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RAMP3 and RAMP1 KO were analyzed with the Caliper Quantum Tx micro CT scanner (Perkin Elmer) located at the Zurich Integrative Rodent Physiology core facility (ZIRP, UZH) using the settings (50 kV, 160 mA, 60 mm field of view). Similar to the RAMP1/3 KO mice, the region between L1 and L4 were evaluated using the Analyze 12.0 Software (AnalyzeDirect Inc, KS, USA), allowing subcutaneous and visceral fat mass, as well as lean mass, to be distinguished. In addition to LBM + 0.2FM, the fat ratio was calculated (subcutaneous + visceral fat)/(subcutaneous + visceral fat + lean mass).

Immunohistochemistry

Brain perfusion. Mice and their respective WT littermates were fasted for 12 h and at dark onset were injected with vehicle (NaCl 0.9%) or amylin (i.p. 50 μg/kg). 90 min post-injection, mice were anesthetized (Pentobarbital 100 mg/kg, i.p.) and perfused for 1.5 min with 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB-PFA, pH 7.4) for 2.5 min. Brains were post-fixed overnight in 4% PB-PFA and cryoprotected overnight in 20% sucrose-0.1 M PB. They were then frozen in hexane on dry ice for 3 min and stored at −80 °C. Frozen caudal hindbrains were cut at 25 μm in 4 series and were mounted onto superfrost plus slides (Life Technologies Europe, Zug, Switzerland). Slides were stored in cryoprotectant (50% 0.02 M KPBS, 30% ethylene glycol, 20% glycerol) at −20 °C until staining for amylin-induced c-Fos and CTR fibers immunohistofluorescence (IHF) in the AP.

c-Fos IHF. Slide mounted sections were washed in 0.02 M KPBS before being blocked overnight with 5% normal donkey serum (NDS), 0.4% Triton X-100 in 0.02 M KPBS at 4 °C. Single-label c-Fos IHF was carried out using rabbit anti-c-Fos antibody (1:500; 2250, Cell Signaling Technologies, BioConcept, Allschwil, Switzerland) and slides were incubated for 48 h at 4 °C. Slides were washed in 0.02 M KPBS 5 times for 5 min and incubated for 2 h with 488 donkey anti-rabbit for 2 h (1:100, Jackson ImmunoResearch, LubioScience, Luzern, Switzerland). Sections were then counterstained with DAPI, and coverslipped using Vectashield Hardset mounting medium (Vectorlabs, Servion, Switzerland).

Calcitonin receptor IHF. Slide mounted sections were washed in 0.1% PBST (0.1% Triton X-100 in 0.01 M PBS) before being blocked for 90 min with 3% NDS, 0.3% Triton X-100 in 0.01 M PBS. Slides were washed in 0.1% PBST and incubated for 48 h at 4 °C in rabbit anti-CTR (1:1000, ab11042, Abcam, Cambridge, UK) in 0.3% PBST. Slides were washed in 0.01 M PBS and in 0.1% PBST. Secondary antibody (CY3 donkey anti-rabbit 1:100 in 0.3% PBST, Jackson ImmunoResearch) was then applied for 2 h (Duffy et al., 2018; Potes et al., 2012). Finally, sections were washed in 0.01 M PBS, counterstained with DAPI, and coverslipped using Vectashield Hardset mounting medium.

Quantitative analysis of immunolabelled cells and fibers. Cells expressing c-Fos in the AP were imaged using an L2 Imager upright microscope (Zeiss, Germany). Images containing AP were set to the same threshold and c-Fos-positive neurons were counted using ImageJ (NIH) to allow for quantification. Three consecutive AP sections from bregma −7.31 to −7.55 (Paxinos and Franklin, 2012), as verified by the DAPI counterstain, were used for quantification.

For the quantitative analysis of CTR fiber density, three sections through the AP from animals of each experimental group were acquired using a Zeiss SP8 confocal system equipped with a 20×/0.75 objective (HD1 488, laser 2% with 10% gain, zoom 1, pinhole 1, Z stack 21 μm, step of 1 μm). Slides were numerically coded to obscure the treatment group. Image analysis was performed using ImageJ analysis software (NIH) as previously described (Lutz et al., 2018). Briefly, each image plane was set to a threshold and binarized to isolate labelled fibers from the background and to compensate for differences in fluorescence intensity. The integrated intensity, which reflects the total number of pixels in the binarized image, was then calculated. This procedure was conducted for each image plane in the stack, and the values for all of the image planes in a stack were summed. The resulting value is an accurate index of the density of the processes in the volume sampled. To ensure similar imaging conditions, the same microscope set-up (including objective, zoom, laser power, gain) was used to acquire all images within the same experiment. For representative images, images were equally adjusted for brightness and contrast within the same experiment.

Statistics

Statistical comparisons among variables were made by 1- or 2-way ANOVA with repeated measure and by 3-way ANOVA (factors: time, genotype and sex), as appropriate, with multiple comparison post-hoc analysis. Comparisons between control and treated groups were assessed using *t*-test for nonparametric statistics (GraphPad Prism, La Jolla, CA, USA). All data are expressed as mean ± SEM.

RESULTS

Role of RAMP1 and 3 in regulating body weight, food intake, body composition and glucose tolerance

Body weight, food intake, glucose tolerance and plasma hormones on chow and 45% HF diet of male RAMP1/3 KO mice (cohort 1). RAMP1/3 KO and WT male mice gained the same amount of weight and ate similar amounts of food on chow diet (Fig. 1A–C). After 4 wks on 45% HF diet, RAMP1/3 KO gained more weight than WT mice and cumulative food intake started to significantly differ at the sixth week on 45% HF diet

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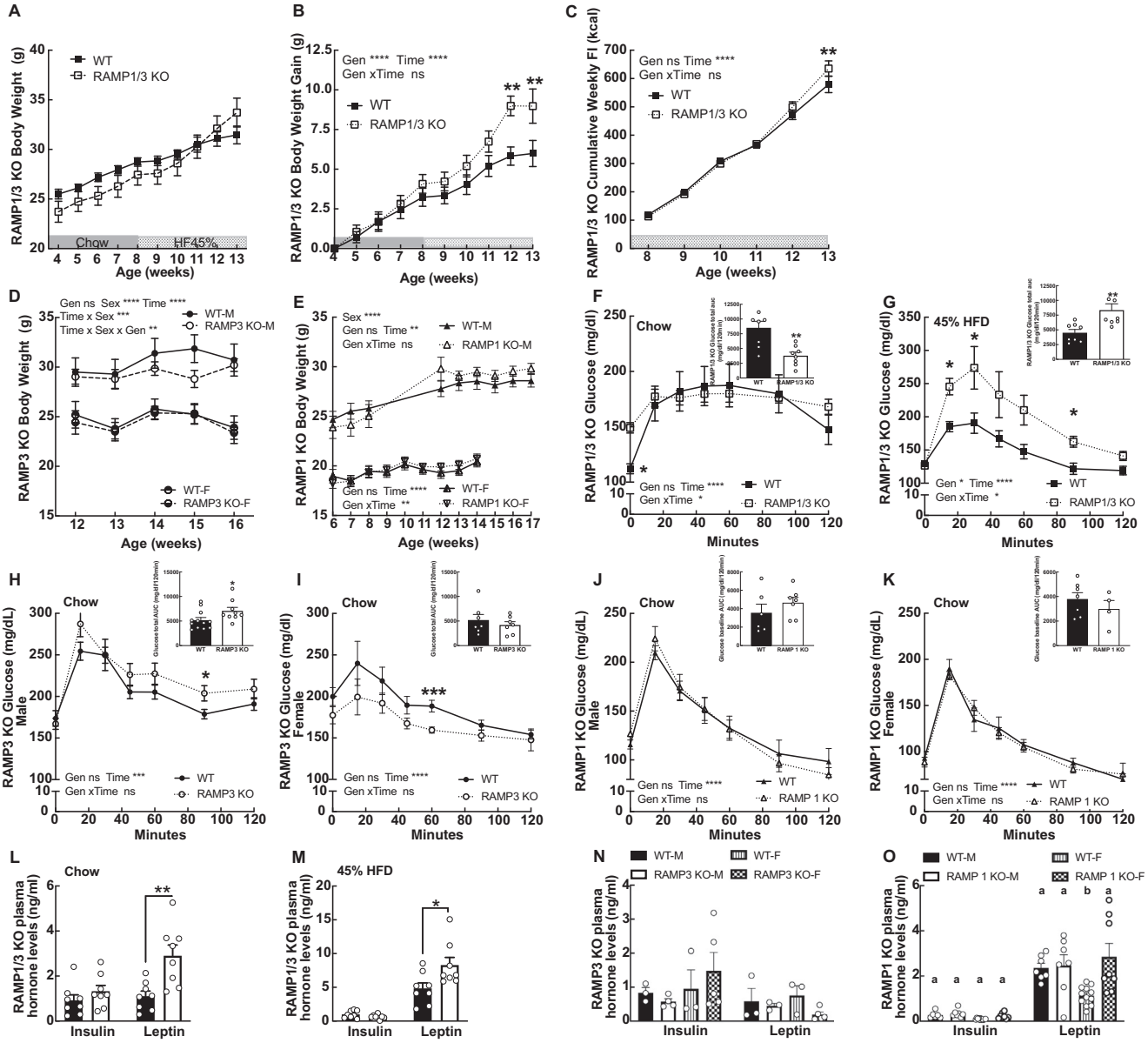

Fig. 1. Body weight, OGTT and blood analysis of WT/RAMP1/3 KO on chow and 45% HF diet, WT vs. RAMP1/3 KO (cohort 1 and 3), RAMP3 KO (cohort 1), and RAMP1 KO (cohort 1–2), on chow diet. Body weight (**A, D, E**), body weight gain (**B**), food intake (**C**), OGTT and AUC on chow diet (**F**, **H, I, J, K**) and 45%HF diet (**G**) in 15 wk-old male WT and RAMP 1/3 KO mice after 4 wks on chow diet and 7 wks on 45%HF diet, 12 to 16 wk-old WT and RAMP3 KO male (M) and female (F) mice, 6 to 17 wk-old WT and RAMP1 KO male and female mice. Baseline insulin and leptin measurements were performed at the end of each diet period (**L–O**). Values are represented as mean ± SEM; n = 8/group for WT and RAMP1/3 KO, n = 8/group for male WT/RAMP3 KO, n = 7/group for female WT/RAMP3 KO and n = 8/group for male and female WT/RAMP1 KO. Statistics: two-way ANOVA with repeated measure followed by post-hoc Sidak's test or Student's t-test. Factors: genotype (Gen) and time. Data with differing superscript differ from each other at $P < 0.05$. *$P < 0.05$, **$P < 0.01$ WT vs. KO.

($P < 0.01$, Fig. 1A–C). Body composition analysis on chow diet was not different between 13 wk-old WT and RAMP1/3 KO mice (Fig. 2Q). After 7 weeks on 45% HF diet, RAMP1/3 KO male mice exhibited higher visceral (+53%, $P < 0.05$), subcutaneous (+40%, $P < 0.001$), and total fat mass (+49%, $P < 0.001$, Fig. 2R) while lean mass was not different from WT mice (Fig. 2R). Fasted glucose levels were significantly higher in RAMP1/3 KO male mice than in WT mice fed chow diet ($P < 0.05$, Fig. 1F). The glucose profile during the OGTT was not different between chow-fed WT and RAMP1/3 KO, however, the RAMP1/3 KO area under the curve (AUC, calculated from above baseline) was lower than WT, mainly due to the fact that RAMP1/3 KO mice had a higher baseline glucose value and that the glucose levels only mildly increased after the glucose load. On 45% HF diet, RAMP1/3 KO and WT male mice had similar levels of fasted blood glucose (Fig. 1G). However, after 7 weeks on 45% HF diet, RAMP1/3 KO mice became glucose intolerant ($P < 0.05$, Fig. 1G) with a 103% increase in AUC ($P < 0.01$, Fig. 1G). On chow and 45% HF diet, baseline insulin levels were not

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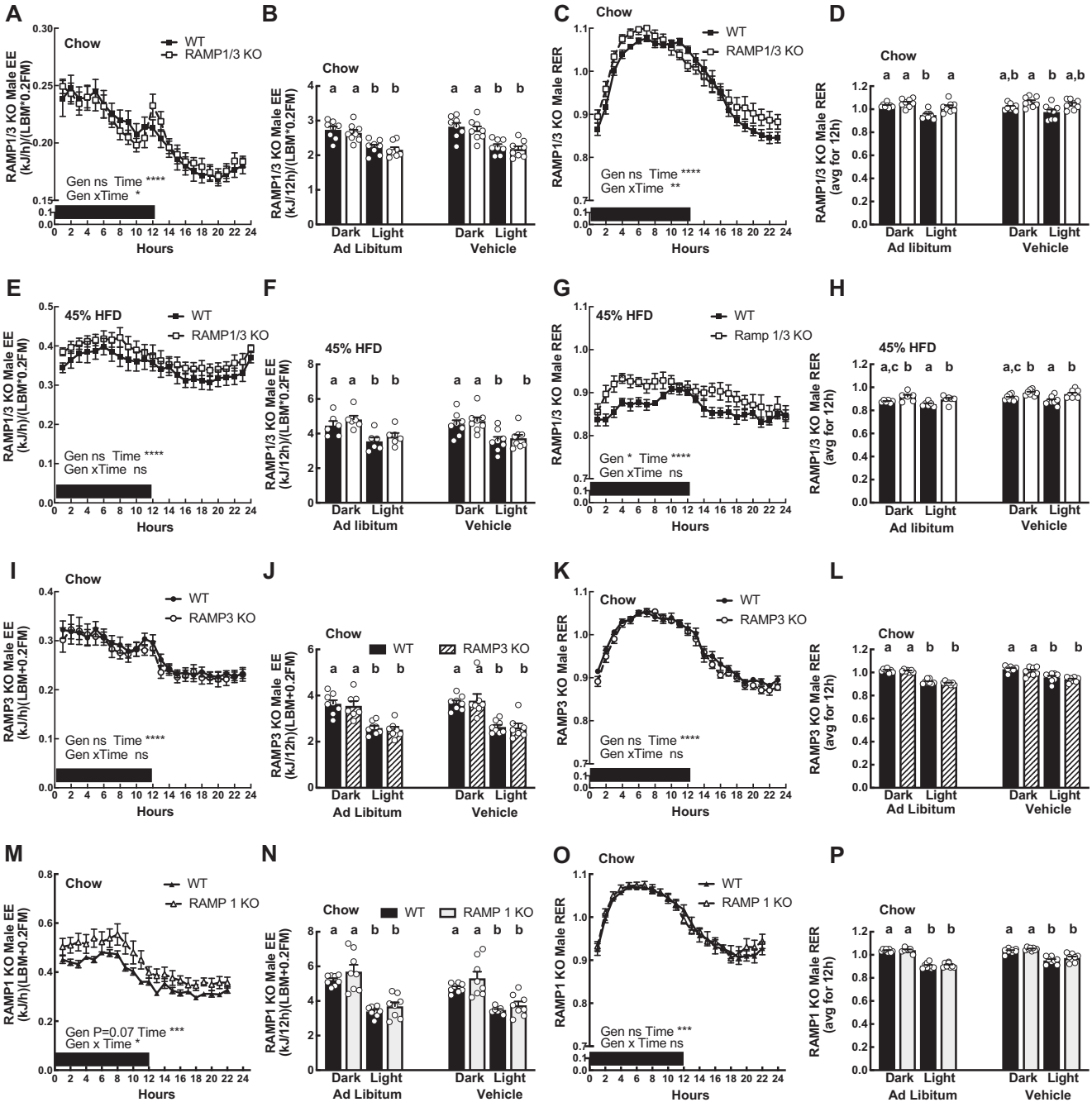

Fig. 2. Energy expenditure, RER and body composition of male WT/RAMP1/3 KO, on chow (cohort 3) and 45% HF diet (cohort 1), WT/RAMP3 KO (cohort 2), and WT/RAMP1 KO (cohort 1–2) on chow diet. 24 h Energy expenditure (EE in kJ/h normalized to LBM *0.2FM) (**A, E, I, M**) and respiratory exchange ratio (RER) (**C, G, K, O**) were measured in *ad libitum* fed mice. EE (**B, F, J, N**) and RER (**D, H, L, P**) during the light and the dark phase were averaged in *ad libitum* or after a 12 h fast followed by vehicle injection (i.p. NaCl 0.9%) in chow (**A–D**) and 45% HF diet (**E–H**) fed in male WT and RAMP 1/3 KO mice, chow-fed male WT and RAMP3 KO mice (**I–L**), chow-fed male WT and RAMP1 KO mice (**M–P**). Body composition was determined between lumbar vertebrae 1 and 4 (L1-L4) in all genotype (**Q–T**). Values are represented as mean ± SEM; n = 8/group for WT and RAMP1/3 KO, WT/RAMP3 KO and WT/RAMP1 KO. Statistics: two-way ANOVA with repeated measure followed by post-hoc Sidak's test or Student's t-test. Factors: genotype (Gen) and time. Data with differing superscript differ from each other at $P < 0.05$. *$P < 0.05$, ***$P < 0.001$ WT vs. KO.

different, while leptin levels in RAMP1/3 KO mice were significantly increased compared to WT mice ($P < 0.01$, Fig. 1L, M). Furthermore, RAMP1/3 KO mice exhibited elevated leptin levels on 45% HF diet, compared to chow, which corresponds with the increase in fat mass in HF diet-fed RAMP1/3 KO mice ($P < 0.001$, Fig. 2R).

Body weight, glucose tolerance and plasma hormones on chow diet of male and female RAMP3 KO and RAMP1 KO mice. RAMP3 KO and WT littermate mice displayed the same body weight on chow diet; generally, male mice displayed a higher body weight than female mice ($P < 0.0001$, $F(1, 44) = 21.60$, Fig. 1D). RAMP3 KO

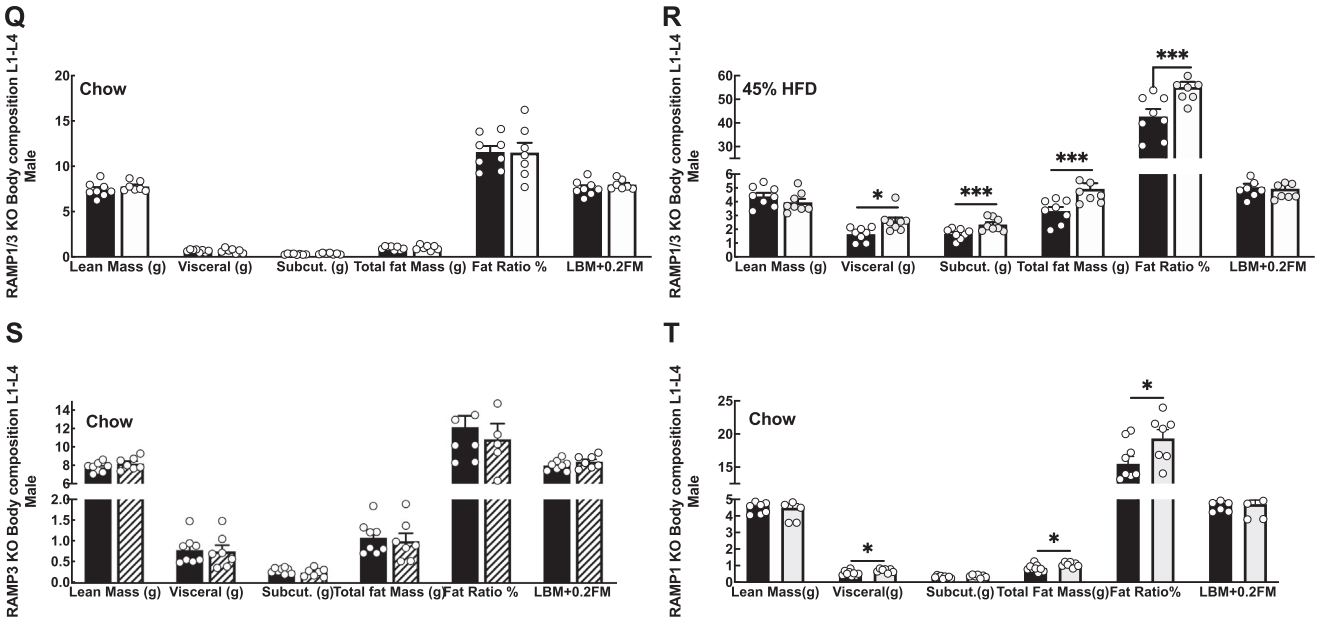

Fig. 2 (*continued*)

and WT mice showed no difference in lean and fat mass *(cohort 2;* Fig. 2S). Male and female RAMP3 KO displayed comparable fasted glucose levels compared to their respective WT littermates (*cohort 2;* Fig. 1H, I). Compared to WT mice, male RAMP3 KO mice displayed delayed glucose clearance during the OGTT and a higher AUC (*cohort 2;* $P < 0.05$, Fig. 1H), suggesting that the male RAMP3 KO mice are glucose intolerant. In contrast, an improvement in glucose tolerance was observed in RAMP3 KO female mice (Fig. 1I). Circulating levels of insulin and leptin were not different between male or female RAMP3 KO and WT littermates fed chow diet (Fig. 1N).

Both male and female RAMP1 KO mice displayed the same body weight on chow diet as their WT littermates (*cohort 1–2;* Fig. 1E); female mice were consistently lighter than male mice ($P < 0.0001$, $F(1, 37) = 43.72$, Fig. 1D). Contrary to RAMP3 KO mice, RAMP1 KO male mice on chow diet had higher visceral fat ($P < 0.05$), total fat mass ($P < 0.05$), and displayed a higher fat ratio ($P < 0.05$) than their WT littermates (*cohort 1–2;* Fig. 2T), but had similar leptin levels (*cohort 1–2;* Fig. 1O). Body composition of the female mice was not different between WT and RAMP1 KO (Data not shown), despite elevated leptin levels in the RAMP1 KO female mice (+131%, $P < 0.01$, Fig. 1O). Similar to RAMP3 KO mice, fasted glucose levels were not different in RAMP1 KO and WT mice (*cohort 1–2;* Fig. 1J, K). WT and RAMP1 KO mice also displayed a similar glucose profile during the OGTT (*cohort 1–2;* Fig. 1J, K), however female mice had an improved glucose tolerance compared to male mice ($P < 0.0014$, $F(1, 24) = 13.13$, Fig. 1J, K), which is likely due to their lower body weight. Baseline insulin levels were not different across all groups (*cohort 1–2;* Fig. 1O).

Feeding behavior of male and female RAMP1/3 KO, RAMP3 KO and RAMP1 KO mice

Male RAMP1/3 KO mice consumed 38% fewer meals in 24 h that were 51% longer in duration, and 116% larger in size in the light phase compared to WT mice (*cohort 2;* $P < 0.05$, Table 1). The intermeal interval (IMI) was also increased by 67% in the RAMP1/3 KO male mice compared to WT ($P < 0.05$, Table 1). This suggests that *ad libitum*-fed RAMP1/3 KO male mice feel less satiated during a meal, but sated longer after a meal than WT mice. Opposite to male mice, female WT and RAMP1/3 KO ate the same amount of food during the light and dark phases, with no differences in meal number or meal size; only IMI was increased in both the dark and light phase in RAMP1/3 KO females. Even though some meal pattern parameters were significantly different in male and not in female mice, no overall difference between male and female RAMP1/3 KO was detected during the 24-h time-period ($P = 0.85$, $F(1, 78) = 0.0345$). However, when taking into account all food intake parameters, an interaction between time and genotype was observed ($P = 0.03$, $F(3,78) = 3.03$).

On chow diet, *ad libitum*-fed WT and RAMP3 KO mice showed no differences in 24-h food intake, meal number, meal size or duration. In male RAMP3 KO mice, the average 24-h IMI was decreased by 34 % compared to WT, resulting from a decrease in the light phase (*cohort 1;* $P < 0.05$, Table 2). Female RAMP3 KO mice displayed a significant decrease in total food intake, meal size and IMI compared to WT mice, but only in the light phase (*cohort 1;* $P < 0.05$, Table 2). Overall male and female 24-h food intake pattern was different ($P < 0.01$, $F(1, 24) = 7.618$) but no genotype effect was detected ($P = 0.59$, $F(1, 24) = 0.29$). During the light phase only, there was an overall difference

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Table 1. *Ad libitum* food intake, meal number, meal size, meal duration and intermeal interval (IMI) for 24 h and during the light and dark phase in male and female WT vs. RAMP1/3 KO mice (cohort 2). Values are represented as mean ± SEM; n = 6–7/group; *P < 0.05 WT vs. KO after an unpaired t-test

	24 h		12 h light phase		12 h dark phase	
	WT	**RAMP1/3 KO**	**WT**	**RAMP1/3 KO**	**WT**	**RAMP1/3 KO**
Male						
Total food intake (g)	4.61 ± 0.43	4.16 ± 0.39	1.04 ± 0.16	1.33 ± 0.34	3.35 ± 0.43	2.67 ± 0.56
Meal number	12.5 ± 0.75	7.75 ± 1.53*	4.17 ± 0.38	2.63 ± 0.52	7.08 ± 0.60	5.00 ± 0.51
Meal size	0.37 ± 0.03	0.58 ± 0.07	0.25 ± 0.03	0.54 ± 0.02*	0.56 ± 0.11	0.68 ± 0.24
Meal duration (Min)	40.8 ± 4.77	61.9 ± 17.6*	31 ± 2	39 ± 3	74.4 ± 17	96 ± 31*
IMI (Min)	83 ± 4	139 ± 18*	139 ± 19	236 ± 50*	46 ± 6	97 ± 20**
Female						
Total food intake (g)	4.00 ± 0.45	3.80 ± 0.36	1.29 ± 0.33	1.31 ± 0.41	2.57 ± 0.19	2.47 ± 0.26
Meal number	10.6 ± 1.31	10.5 ± 1.65	3.83 ± 0.70	3.61 ± 0.72	6.33 ± 0.75	5.89 ± 0.98
Meal size	0.42 ± 0.05	0.41 ± 0.05	0.35 ± 0.08	0.32 ± 0.06	0.50 ± 0.08	0.53 ± 0.10
Meal duration (Min)	40.2 ± 5.69	56.3 ± 9.1	34 ± 7	51 ± 6	79 ± 19	85 ± 18
IMI (Min)	108 ± 10	118 ± 13	167 ± 33	224 ± 30*	61 ± 8	89 ± 12*

RAMP1/3 KO (24 h) -3 way-ANOVA Time P < 0.0001, Sex NS, Gen NS, Time × Sex NS, Time × Gen P = 0.03, Sex × Gen NS, Time × Sex × Gen NS.

Table 2. *Ad libitum* food intake, meal number, meal size, meal duration and intermeal interval (IMI) for 24 h and during the light and dark phase in WT vs. RAMP3 KO male and female mice (cohort 2) and RAMP1 KO male and female mice (cohort 1). Values are represented as mean ± SEM; n = 6–7/group (RAMP3) and n = 8/group (RAMP1); *P < 0.05 WT vs. KO after an unpaired t-test

	24 h		12 h light phase		12 h dark phase	
	WT	**RAMP3 KO**	**WT**	**RAMP3 KO**	**WT**	**RAMP3 KO**
Male						
Total food intake (g)	5.51 ± 0.34	5.54 ± 0.59	0.60 ± 0.18	0.39 ± 0.06	4.90 ± 0.28	5.15 ± 0.62
Meal number	11.28 ± 1.15	12.14 ± 1.60	3.10 ± 0.46	3.08 ± 0.37	8.15 ± 0.85	9.07 ± 1.20
Meal size	0.46 ± 0.07	0.40 ± 0.06	0.17 ± 0.03	0.12 ± 0.02	0.76 ± 0.13	0.67 ± 0.13
Meal duration (Min)	38 ± 9	30 ± 3.80	10.5 ± 1.70	8.5 ± 0.67	66 ± 16	50 ± 5
IMI (Min)	121 ± 19	74 ± 12*	217 ± 35	137 ± 14*	43 ± 3	39 ± 2
Female						
Total food intake (g)	5.04 ± 0.49	5.91 ± 0.66	0.70 ± 0.10	0.45 ± 0.10*	4.34 ± 0.40	5.46 ± 0.71
Meal number	11.56 ± 1.27	10.5 ± 0.74	3.57 ± 0.38	3.80 ± 0.65	8.00 ± 0.90	6.70 ± 0.20
Meal size	0.46 ± 0.08	0.49 ± 0.06	0.21 ± 0.06	0.12 ± 0.02*	0.72 ± 0.15	0.86 ± 0.12
Meal duration (Min)	41 ± 11	42 ± 3	11 ± 1.5	12.5 ± 2.2	71 ± 12	71 ± 4.5
IMI (Min)	133 ± 20	74 ± 8*	222 ± 35	123 ± 11*	45 ± 7	38 ± 3
	WT	**RAMP1 KO**	**WT**	**RAMP1 KO**	**WT**	**RAMP1 KO**
Male						
Total food intake (g)	3.05 ± 0.41	2.92 ± 0.40	0.52 ± 0.09	0.37 ± 0.10*	2.53 ± 0.35	2.55 ± 0.34
Meal number	18 ± 2	14 ± 2	3.86 ± 0.65	2.63 ± 0.61	14.1 ± 0.87	12.1 ± 0.96
Meal size	0.16 ± 0.02	0.19 ± 0.03	0.14 ± 0.02	0.15 ± 0.02	0.17 ± 0.03	0.21 ± 0.03
Meal duration (Min)	1.86 ± 0.26	2.63 ± 0.43*	1.71 ± 0.50	2.14 ± 0.60	1.86 ± 0.32	2.88 ± 0.40*
IMI (Min)	71 ± 9.3	91.7 ± 12.8*	183 ± 31	239 ± 50	48.3 ± 6.60	69.6 ± 6.20*
Female						
Total food intake (g)	2.47 ± 0.30	2.54 ± 0.32	0.36 ± 0.11	0.41 ± 0.06	2.11 ± 0.29	2.13 ± 0.28
Meal number	19.8 ± 2.95	19 ± 2.5	3.50 ± 0.78	4.38 ± 0.74	17.2 ± 1.51	15.9 ± 1.98
Meal size	0.12 ± 0.02	0.13 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.13 ± 0.02	0.14 ± 0.02
Meal duration (Min)	2.13 ± 0.51	2.50 ± 0.39	1.00 ± 0.31	2.00 ± 0.52	2.13 ± 0.51	2.63 ± 0.43
IMI (Min)	67.3 ± 9.50	67.5 ± 8.8	165 ± 28	153 ± 28	49 ± 7.9	49 ± 6.33

RAMP3 KO (24 h) 3 way-ANOVA Time P < 0.0001, Sex P = 0.01, Gen P = 0.59, Time × Sex P < 0.0001, Time × Gen NS, Sex × Gen NS, Time × Sex × Gen NS. RAMP1 KO (24 h) 3 way-ANOVA Time P < 0.0001, Sex P = 0.049, Gen NS, Time × Sex P < 0.0001, Time × Gen P = 0.008, Sex × Gen NS, Time × Sex × Gen P = 0.008.

between WT and RAMP3 KO meal pattern parameters (P = 0.009, $F(1, 24)$ = 7.983). The consistently observed decrease in IMI suggests that male and female RAMP3 KO mice experience less satiety than WT mice.

Meal pattern analysis in RAMP1 KO mice (*cohort 1;* Table 2) showed that male RAMP1 KO mice took longer meals during the 24-h period (+41%, P < 0.05), which primarily resulted from an increase in meal duration during the dark phase (+54%, P < 0.05). 24-h IMI was

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Table 3. Summary of effects of RAMP 1/3, 3 and 1 KO compared to WT mice

	RAMP1/3 KO	RAMP3 KO	RAMP1 KO
Parameters			
Body Weight	Chow: = HFD: +	=	=
Body Composition	Chow: = HFD: + fat	=	Male: + fat Female: =
Food Intake	Chow: = HFD: +	=	=
Meal Pattern	Male: – Meal number + duration, + IMI Female: = No sex difference	Male: –IMI Female: –IMI Sex difference	Male: + duration, + IMI Female: = Sex difference
Glucose Tolerance	Chow: = HFD: – tolerance	males: - tolerance females: + tolerance	=
Basal Leptin	Chow: + HFD: +	=	Male: = Female: +
Basal Insulin	Chow: = HFD: =	=	=
Energy Expenditure	Chow: = HFD: =	=	=
RER	Chow: + in light HFD: +	=	=
FI response to amylin	Overall: – No sex difference	Overall: – No sex difference	Overall: = Sex difference
FI response to sCT	Overall: – No sex difference	Overall: = No sex difference	Overall: = Sex difference
FI response to leptin	Leptin: – Amylin/leptin: delayed effect Female: N/A	= No sex difference	Leptin: =; Amylin/leptin: N/A Sex difference
Amylin-induced c-Fos	–	=	=
CTR	–	–	=

Legend: +: increased compared to WT, –: decreased compared to WT, =: no change compared to WT, N/A: not analyzed.

also increased in male RAMP1KO mice (+30%, $P < 0.05$) compared to WT mice. Female RAMP1 KO showed no alteration in their meal patterns compared to WT mice. However, male and female 24-h food intake pattern was different ($P = 0.04$, $F(1, 136) = 4.261$) and even though no genotype effect was detected ($P = 0.12$, $F(1, 136) = 2.393$), an interaction among time × sex × genotype was detected ($P = 0.008$, $F(4, 136) = 3.545$).

The meal pattern analysis of the three KO mice models reveals that RAMP1/3 KO mice displayed greater alterations in meal patterns, specifically a decrease in meal number and meal duration and an increase in IMI, than the single RAMP KO mice. A clear sex-difference was detected in the single KO mice model but not in RAMP1/3 KO mice.

Role of RAMP1 and 3 in regulating EE and RER

On chow diet, WT and RAMP1/3 KO mice displayed the same EE (Fig. 2A, B) and RER (*cohort 3;* Fig. 2C, D) under *ad libitum* conditions and during refeeding after a 12-h fast. However, during the light phase, RER was elevated by 7% in *ad libitum* fed RAMP1/3 KO mice as compared to WT mice (*cohort 3;* $P < 0.05$, Fig. 2D). On 45% HF diet, WT and RAMP1/3 KO mice displayed no differences in EE under *ad libitum* conditions or during refeeding after a 12 h fast (*cohort 1;* Fig. 2E, F), whereas the RER of *ad libitum* fed RAMP1/3 KO mice was increased during the dark and light phase by 4.5% and 5%, respectively (*cohort 1;* $P < 0.05$, Fig. 2H). This effect of increased RER in the dark and the light phase was also present in the RAMP1/3 KO mice during the refeeding phase after a 12-h fast (4.5% and 7% respectively $P < 0.05$, Fig. 2H). Overall, the consistent increase in RER suggests that RAMP1/3 KO mice preferentially utilize carbohydrate over fat when fed chow or 45% HF diet. The analysis of the EE and RER in *ad libitum* fed mice showed no significant differences between male WT and RAMP3 KO (*cohort 2;* Fig. 2I–L) or between male WT and RAMP1 KO mice on chow diet (*cohort 1–2;* Fig. 2M–P). Interestingly, peripheral treatment with sCT significantly decreased RER in WT mice, but not in RAMP1KO mice ($P < 0.01$ vs. vehicle; Data not shown).

Thus, while the depletion of either RAMP1 or RAMP3 subunit did not affect EE or RER, the depletion of both subunits in RAMP1/3 KO elevated the RER on chow and 45% HF diet.

Amylin's and salmon calcitonin's effect on food intake in male and female RAMP1/3 KO, RAMP3 KO and RAMP1 KO mice

Increasing doses of amylin, after a 12-h fast, had a significant effect on 2 h food intake in WT mice but

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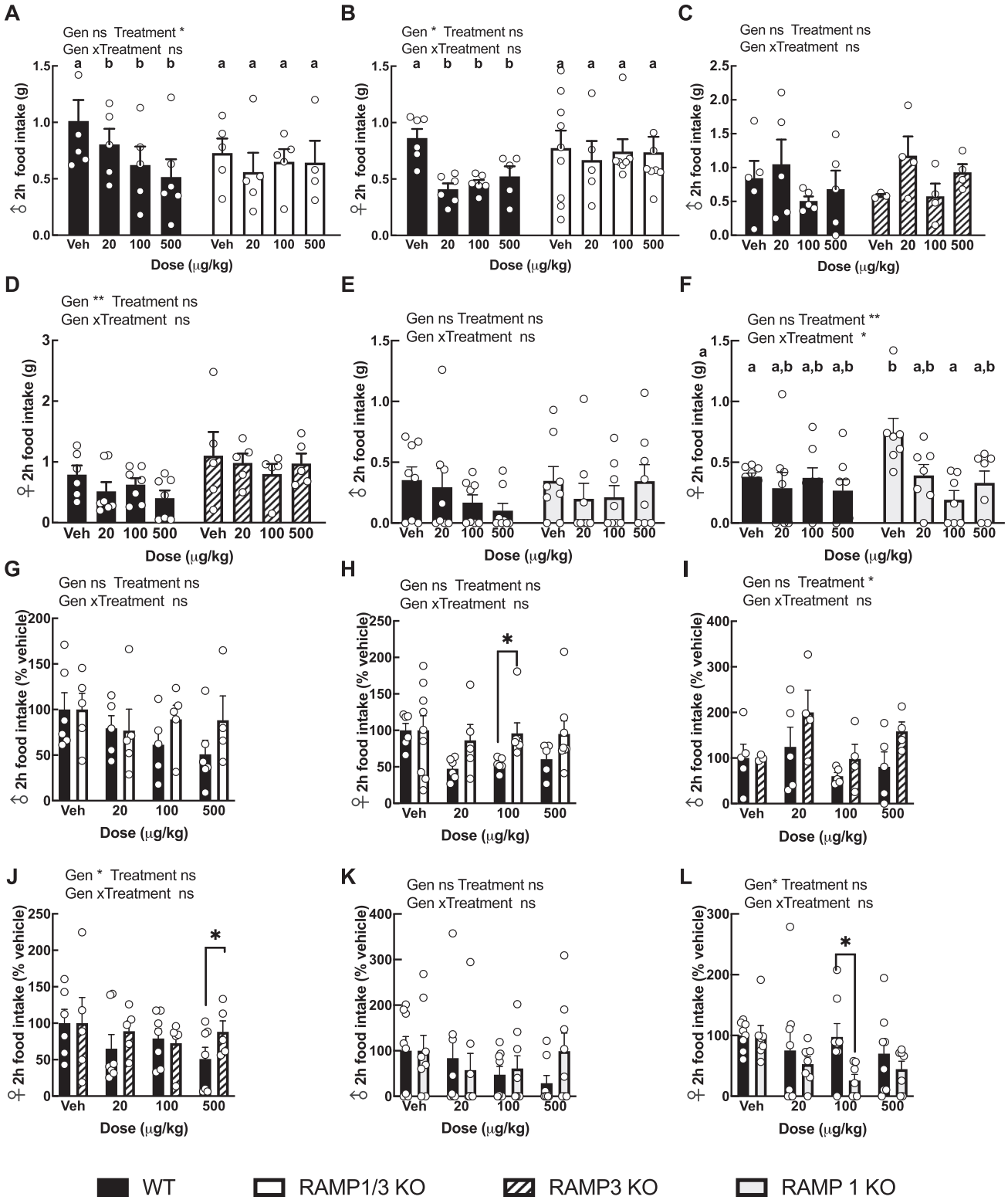

Fig. 3. Amylin- and sCT-induced anorexia test in chow-fed male and female WT vs. RAMP1/3 KO (cohort 2), RAMP3 KO (cohort 1), and RAMP1 KO (cohort 1) mice. Cumulative food intake in g or as a percent of vehicle (veh, NaCl 0.9%) in WT vs. RAMP1/3 KO mice (**A, B, G, H**), WT vs. RAMP3 KO mice (**C, D, I, J**), WT vs. RAMP1 KO mice (**E, F, K, L**) after 2 hours of being injected with either vehicle or amylin (i.p. 20, 100, 500 μg/kg). Mice were also tested for their response to the amylin receptor agonist, salmon calcitonin (sCT 10 μg/kg i.p.) and 2 h food intake was measured in WT vs. RAMP1/3 KO mice (**M, N, S, T**), WT vs. RAMP3 KO mice (**O, P, U, V**), WT vs. RAMP1 KO mice (**Q, R, W, X**). Data are displayed in means of absolute values (**A–F** and **M–R**) and relative to vehicle (**G–L** and **S–X**) ± SEM; n = 6–7/group for WT/RAMP1/3 KO, n = 5–6/group for WT/RAMP3 KO, n = 8/group for WT/RAMP1 KO. Statistics: two-way ANOVA with repeated measure followed by post-hoc Sidak's test. Factors: genotype (Gen) and treatment. (A-F and M–R) Data with differing superscript differ from each other within the same genotype at $P < 0.05$. (G-L and S-X) *$P < 0.05$ WT vs KO. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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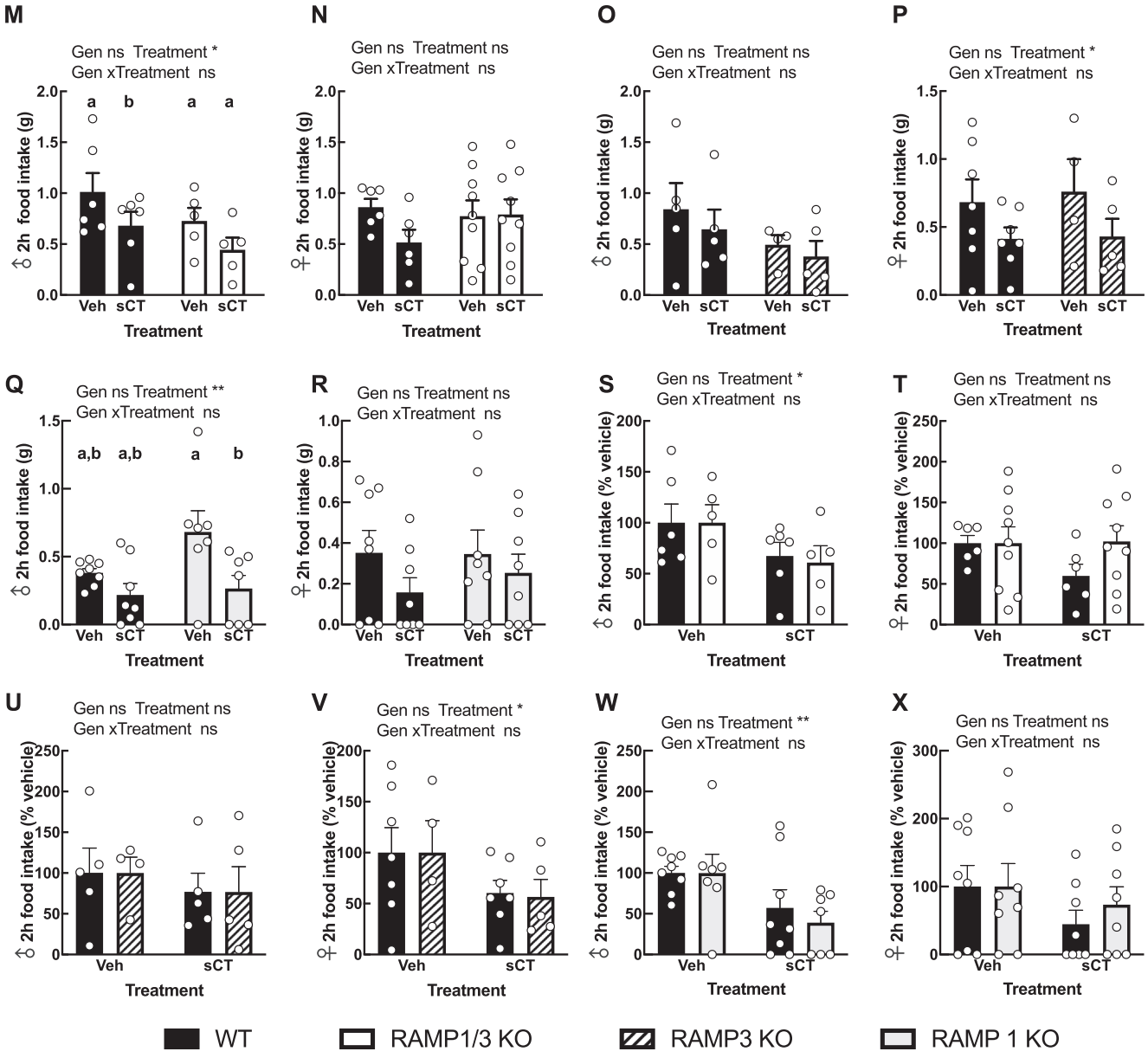

Fig. 3 (*continued*)

showed no effect on RAMP1/3 KO male or female mice; these effects were however lost at the 4 h time point (Figs. 3A, B; 4A, B). When expressed as percent of vehicle, the amylin dose of 500 μg/kg had a stronger effect on 4 h food intake in WT male mice than in RAMP1/3 KO mice ($P < 0.05$, Fig. 4G). Overall, when taking into account all amylin doses at 4 h in male and female mice, a significant interaction genotype by treatment was observed ($P = 0.02$, $F(3, 54) = 3.347$) but no sex difference was observed ($P = 0.09$, $F(1, 21) = 3.125$ Fig. 4A, B). sCT had a stronger anorectic effect at 2 h and 4 h post injection in WT mice than in RAMP1/3 KO male mice ($P = 0.05$, $F(1, 18) = 4.188$ Fig. 3M, $P = 0.018$, $F(1, 18) = 6.654$ Fig. 4M) and a significant difference in genotype between WT and RAMP1/3 KO was detected ($P = 0.028$, $F(1, 18) = 5.705$, Fig. 4M). However, at 24 h, sCT tended to have the same effect in WT and KO mice ($P < 0.05$, data not shown). When expressed as a percent of vehicle, no significant difference was found between WT and RAMP1/3 KO mice (Figs. 3S, T, 4S, T). Similar to male mice, female RAMP1/3 KO did not respond to amylin at the 2 h time point (Fig. 3B, H), and no significant effect of sCT on food intake was observed at 2 h and 4 h (Figs. 3N, T, 4N, T). Together, these data showed that RAMP1/3 KO male and female mice are insensitive to amylin's effect on eating compared to WT mice.

Increasing doses of amylin after a 12 h fast decreased 4 h food intake in a dose response manner in WT female mice, but had no such effect in RAMP3 KO female mice (*cohort 1;* $P = 0.007$, $F(1,39) = 7.9$ Fig. 4D). A

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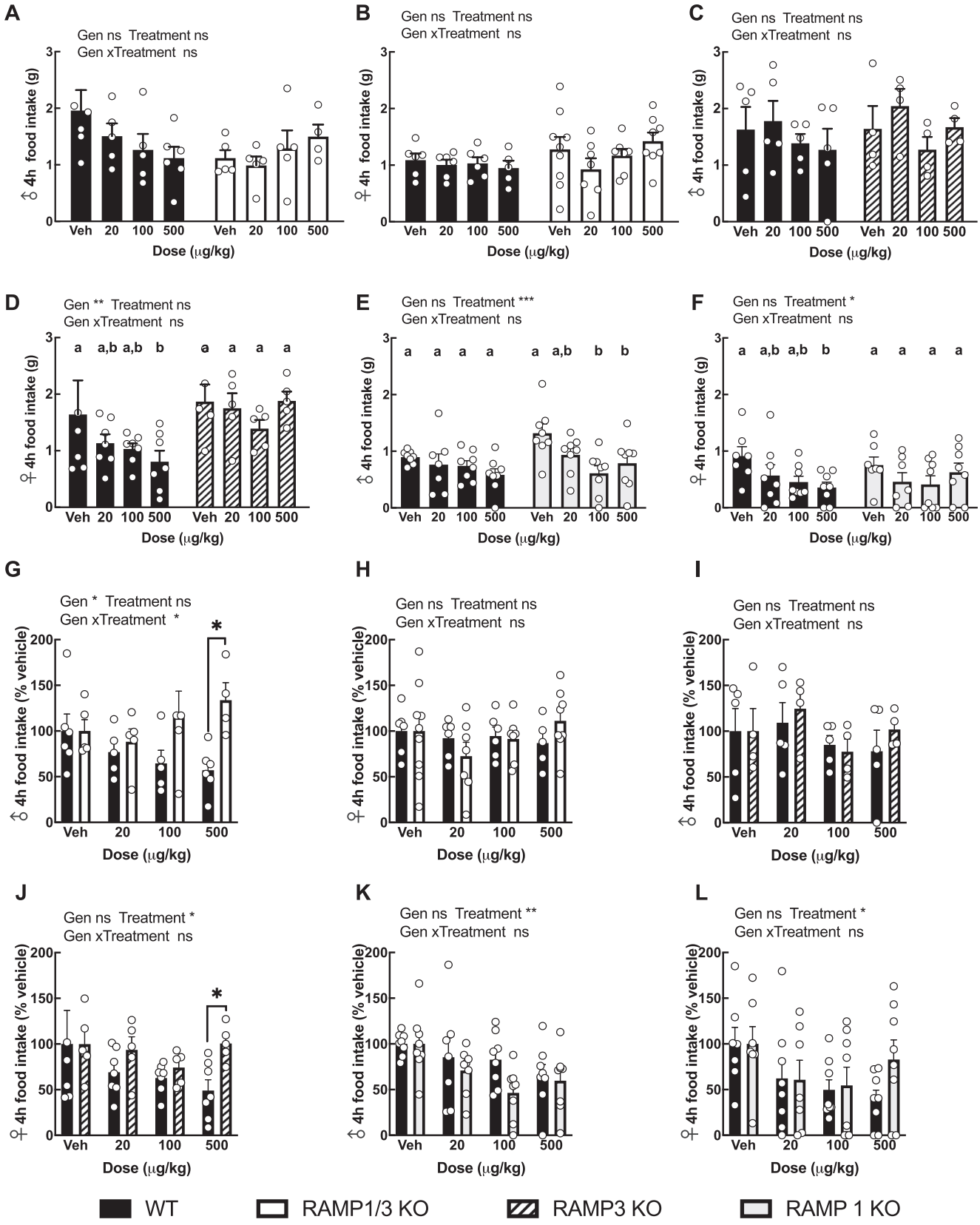

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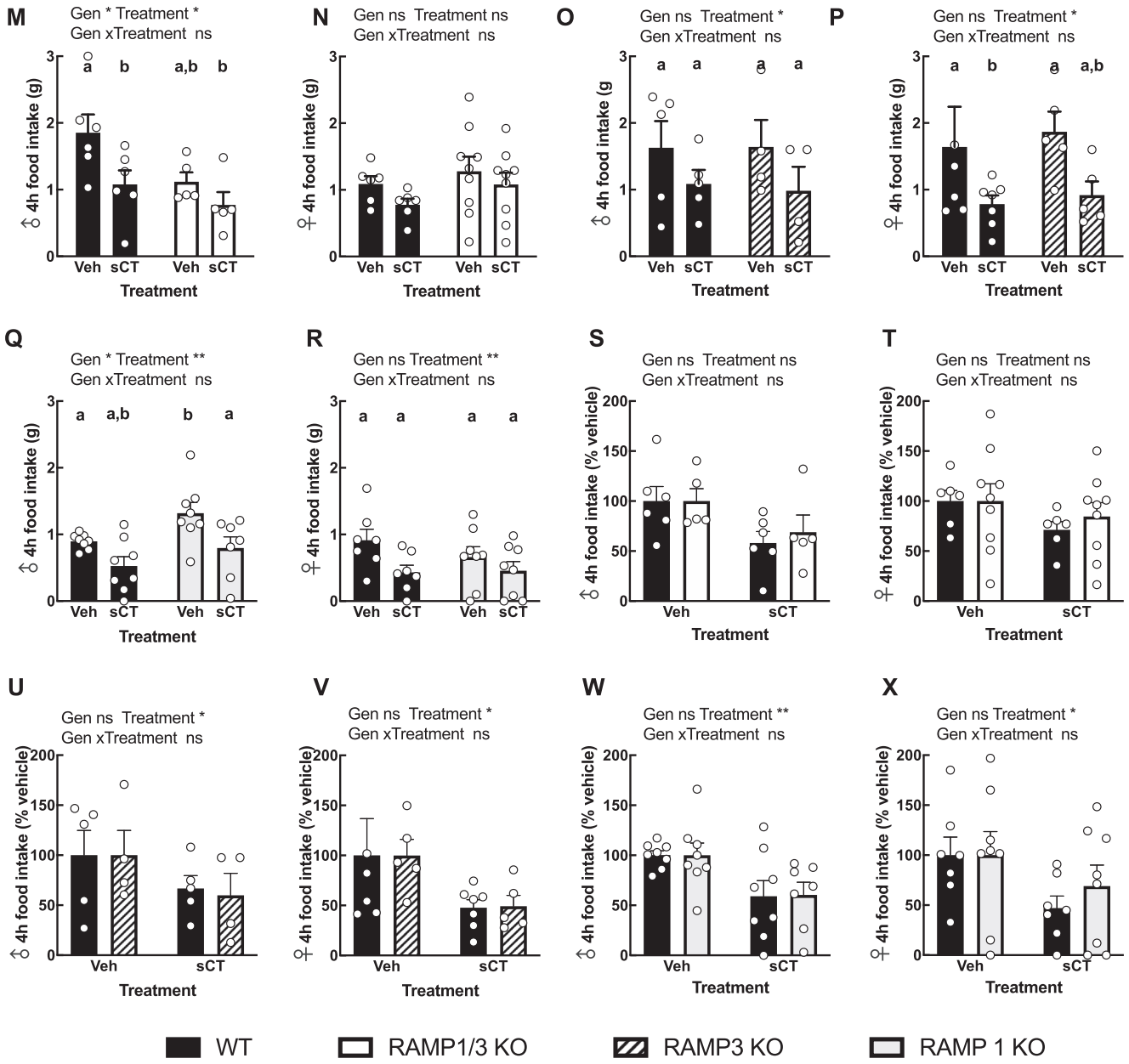

Fig. 4 (*continued*)

significant main effect of genotype was observed in female mice ($P = 0.0052$, $F(1,39) = 8.746$) Figs. 3D, 4D), however, no sex-difference was detected at 2 h or 4 h post-injection ($P = 0.51$, $F(1,14) = 0.43$). When expressed as percent of baseline, 500 μg/kg of amylin had a significantly stronger effect on 2 h and 4 h food intake in WT than in RAMP3 KO female mice (*cohort 1;* $P < 0.05$, Figs. 3J and 4J). sCT significantly decreased food intake at 2 h and 4 h post-injection in WT mice and RAMP3 KO mice (*cohort 1;* $P < 0.05$, Figs. 3P, 4O,

Fig. 4. Amylin- and sCT-induced anorexia test in chow-fed male and female WT vs. RAMP1/3 KO (cohort 2), RAMP3 KO (cohort 1), and RAMP1 KO (cohort 1) mice. Cumulative food intake in g or as a percent of vehicle (veh, NaCl 0.9%) in WT vs. RAMP1/3 KO mice (**A, B, G, H**), WT vs. RAMP3 KO mice (**C, D, I, J**), WT vs. RAMP1 KO mice (**E, F, K, L**) after 4 hours of being injected with either vehicle or amylin (i.p. 20, 100, 500 μg/kg). Mice were also tested for their response to the amylin receptor agonist, salmon calcitonin (sCT 10 μg/kg i.p.) and 4 h food intake was measured in WT vs. RAMP1/3 KO mice (**M, N, S, T**), WT vs. RAMP3 KO mice (**O, P, U, V**), WT vs. RAMP1 KO mice (**Q, R, W, X**). Data are displayed in means of absolute values (**A–F** and **M–R**) and relative to vehicle (**G–L** and **S–X**) ± SEM; n = 6–7/group for WT/RAMP1/3 KO, n = 5–6/group for WT/RAMP3 KO, n = 8/group for WT/RAMP1 KO. Statistics: two-way ANOVA with repeated measure followed by post-hoc Sidak's test. Factors: genotype (Gen) and treatment. (**A–F** and **M–R**) Data with differing superscript differ from each other within the same genotype at $P < 0.05$. (**G–L** and **S–X**) *$P < 0.05$ WT vs KO. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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4P). sCT had a comparable effect in male and female mice ($P = 0.96$ $F(1,27) = 0.0002$). Thus, while amylin was not able to produce an anorectic effect in RAMP3 KO mice, sCT acted normally in male and female RAMP3 KO mice.

In the RAMP1 cohort, amylin similarly decreased 4 h food intake in male and female WT and RAMP1 KO mice (*cohort 1;* treatment $P < 0.001$, $F(3,41) = 6.77$ Fig. 4E and $P < 0.024$, $F(3,39) = 3.49$ Fig. 4F). The overall response to amylin injections was significantly different between male and female RAMP1 KO mice ($P = 0.007$, $F(1,28) = 8.42$ Fig. 4E, F). No genotype difference regarding amylin's anorectic response was detected between WT and RAMP1 KO mice at either 2 h or 4 h. The anorectic response to sCT was not different between WT and RAMP1 KO male mice 4 h post-injection (*cohort 1;* treatment $P = 0.004$, $F(1,13) = 12.5$, genotype $P = 0.029$, $F(1,14) = 5.90$ Fig. 4Q) and a main treatment effect was detected in female WT and RAMP1 KO mice ($P = 0.006$, $F(1,13) = 10.4$ Fig. 4R). A main effect of sex was detected in RAMP1 KO mice in response to sCT ($P = 0.02$, $F(1,27) = 5.877$). These data indicate that both amylin and sCT are able to produce an anorectic effect in RAMP1 KO mice, suggesting that RAMP3, but not RAMP1, subunit is necessary for amylin's anorectic effect.

Leptin-induced anorexia in male and female mice RAMP1/3 KO, RAMP3 KO and RAMP1 KO mice

Leptin injection in WT mice tended to decrease food intake at 4 h as expected, whereas it had no effect in RAMP1/3 KO mice (*cohort 3;* Treatment effect $P = 0.001$, $F(2, 34) = 8.071$, Fig. 5A). The combination of amylin and leptin induced a further reduction in food intake at 4 h post-injection, but this only reached statistical significance in the WT mice and not in RAMP1/3 KO mice ($P < 0.05$, Fig. 5A). When expressed as a percent of vehicle, the leptin and amylin combination had a 56% stronger decrease on food intake at 1 h in WT mice as compared to KO mice ($P < 0.05$, Data not shown). Thus, while leptin or amylin alone had no effect on RAMP1/3 KO mice, their synergistic action on food intake was delayed, suggesting that leptin signaling pathway may still be functional in these mice.

In WT and RAMP3 KO male mice, leptin alone had no effect on food intake at any time point (*cohort 2;* Fig. 5C). Administration of amylin and leptin together decreased food intake by approximately 50% in both WT and RAMP3 KO male mice 4 h after injection (Treatment effect $P < 0.01$ $F(2, 45) = 7.178$, Fig. 5D), however this treatment only reduced food intake compared to vehicle rather in WT male mice than in RAMP3KO male mice (Fig. 5B). In female mice, no treatment effect was detected in WT or RAMP3 KO mice at 4 h post injection (Fig. 5E). When expressed as a percent of baseline, leptin had a significantly stronger anorectic effect in WT mice than in RAMP3 KO female mice ($P < 0.05$, Fig. 5F). Overall, no sex-difference between WT and RAMP3 KO mice was detected for their response to leptin or leptin + amylin ($P = 0.40$ $F(1, 26) = 0.7051$). Thus, the absence of RAMP3 seems to not interfere with leptin signaling in male mice while female mice seem to be unresponsive.

WT and RAMP1 KO mice presented an overall sex-difference for their response to leptin (*cohort 2;* $P = 0.0025$, $F(1, 28) = 11.01$). However, leptin did not produce an anorectic effect at any time point (*cohort 2;* Fig. 5G–J) regardless of sex or genotype. Unfortunately, the combination of amylin and leptin was not assessed in these mice.

Effect of amylin on c-Fos and CTR fiber density in the AP

Amylin significantly increased the number of c-Fos positive neurons in the AP of WT mice by 7-fold (*cohort 2–3;* $P < 0.05$, Fig. 6A, C) compared to saline control mice while it had no effect in RAMP1/3 KO male mice. CTR fiber density in the AP was also decreased by 80% in the RAMP1/3 KO mice compared to WT male mice (*cohort 3;* $P < 0.05$, Fig. 6B, C). This decrease in c-Fos and CTR in RAMP1/3 KO may explain the absence of an amylin effect on food intake (Fig. 4A, G). RAMP1/3 KO female mice were not assessed.

In contrast to the double KO mice, amylin significantly increased the number of c-Fos positive neurons in the AP of both male and female WT and RAMP3 KO mice by 14-fold and 12-fold, respectively (*cohort 1;* $P < 0.05$, Fig. 6D, F). Similarly, amylin induced a 6-fold increase in c-Fos in male and female WT and RAMP1 KO mice (*cohort 1–2;* $P < 0.001$, Fig. 6G, H). CTR fiber density in the AP, however, was decreased by 72% and 55% in male and female RAMP3 KO mice, respectively, compared to WT mice (*cohort 1;* $P < 0.05$, Fig. 6E, F). In RAMP1 KO mice, CTR fiber density in the AP was comparable to their WT counterparts (*cohort 1–2;* Fig. 6H, I).

DISCUSSION

These studies were undertaken to assess the influence of deficiency in RAMP1, RAMP3, or RAMP 1 and 3 on whole-body energy homeostasis using global knockout mice. RAMPs are a critical component of the amylin receptor, they promote receptor specificity and enhance amylin's affinity to the CTR core (Christopoulos et al., 1999). Therefore, this study was particularly focused on whether amylin or its receptor agonist, sCT, preferentially act on specific amylin receptor subtype to affect food intake. By phenotyping these three different mouse models, we highlighted the role of the RAMP1 subunit as a mediator of fat storage and utilization, while the RAMP3 subunit contributes to glucose homeostasis and amylin's anorectic effect. Finally, the presence of at least one RAMP subunit (1 or 3) is both necessary and sufficient for amylin-induced c-Fos in the AP, when CTR is present (Table 3).

The combined depletion of RAMP1 and 3 did not affect body weight in male or female mice maintained on chow diet. However, RAMP1/3 KO male mice maintained on 45% HF diet gained more weight and ate more than WT male mice, resulting in higher fat mass and leptin levels. Our data suggest that RAMP1

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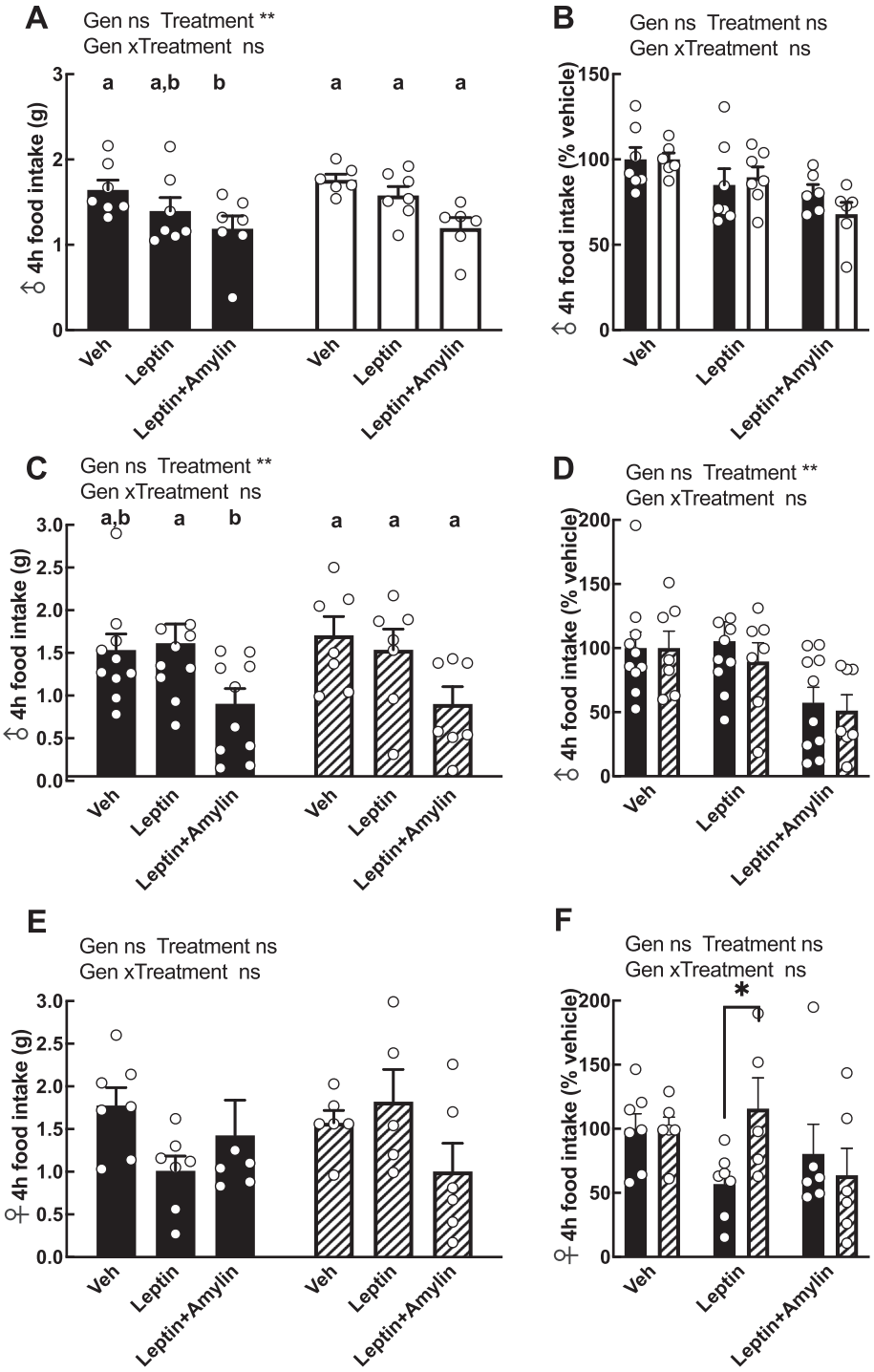

Fig. 5. Leptin- and amylin + leptin-induced anorexia test in chow-fed male WT vs. RAMP1/3 KO (cohort 3), male and female RAMP3 KO (cohort 2) and male and female RAMP1 KO (cohort 2) mice. Cumulative food intake in g or as a percent of vehicle (veh, NaCl 0.9%) in WT vs. RAMP1/3 KO male mice (**A, B**), WT vs. RAMP3 KO male and female mice (**D**–**F**), WT vs. RAMP1 KO male and female mice (**G**–**J**) after 4 hours of being injected with either vehicle or leptin (i.p. 5 mg/kg) or amylin + leptin (i.p. 50 μg/kg + 5 mg/kg). Data are displayed in means of absolute values (**A, C, E, G, I**) and relative to vehicle (**B, D, F, H, J**) ± SEM; n = 8/group for WT/RAMP1/3 KO, n = 8/group for WT/RAMP3 KO, n = 8/group for WT/RAMP1 KO. Statistics: two-way ANOVA with repeated measure followed by post-hoc Sidak's test. Factors: genotype (Gen) and treatment. Data with differing superscript differ from each other within the same genotype at $P < 0.05$. *$P < 0.05$ WT vs KO. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

deficiency, and not RAMP3, drives this effect, as the male RAMP1 KO mice also demonstrated increased fat mass, while RAMP3 KO mice did not. While female RAMP1 KO mice showed no change in fat mass, they exhibited increased leptin levels compared to WT mice. Despite the increased fat mass in RAMP1 KO and HF diet-fed RAMP1/3 KO, neither genotype demonstrated a compensatory increase in EE. The RAMP1/3 KO mice did, however, exhibit preferred utilization of carbohydrates over fat. Chronic central injection of amylin was shown to decrease RER, which indicates higher fat utilization, in outbred male rats (Wielinga et al., 2010); hence, the lack of amylin signaling in the RAMP1/3 KO mice could be the cause of elevated RER. As with the fat mass, RAMP1 appears more critical for this effect on RER; while baseline RER was similar in WT and RAMP1KO, sCT was unable to decrease RER in RAMP1 KO compared to WT mice. Thus, when fats are not properly utilized, this could lead to the excessive accumulation that was observed in the RAMP 1/3 and 1 KOs, leading to the idea that RAMP1 is necessary to promote fat utilization. In agreement with this, the overexpression of human RAMP1 (hRAMP1) in neurons induced a lean phenotype and lower leptin levels due to higher EE, increased oxygen consumption, body temperature, and sympathetic tone subverting brown adipose tissue (BAT) in mice (Fernandes-Santos et al., 2013). Further, nestin/hRAMP1 transgenic mice displayed elevated mRNA levels of peroxisome proliferator–activated receptor γ coactivator 1α and uncoupling protein 1 and 3 in BAT, and these changes were reversed by chronic blockade of sympathetic nervous system signaling (Zhang et al., 2011). In line with these previous studies, our results indicate that RAMP1 contributes to fat utilization, potentially

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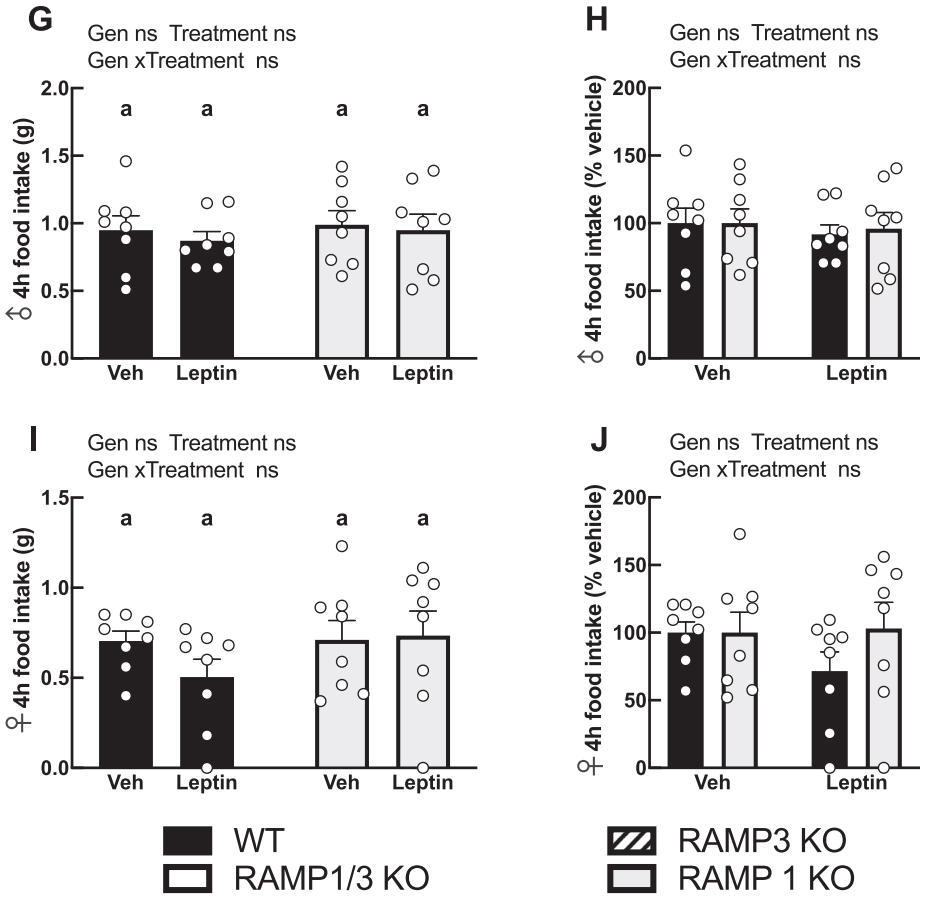

Fig. 5 (*continued*)

through the activation of the sympathetic nervous system. Further experiments testing sympathetic activity in RAMP1 KO mice are required to validate this hypothesis.

In addition to increased weight gain and fat mass, RAMP1/3 KO mice maintained on HF diet were glucose intolerant. Male RAMP3 KO mice were also glucose intolerant, despite displaying similar body weight and fat mass to WT mice, suggesting that RAMP3 contributes to the regulation of glucose homeostasis, independently of changes in body weight or composition. Supporting this finding, Liu et al. also described a tendency for decreased glucose tolerance in female RAMP3 KO (Liu et al., 2018). The fact that male and female RAMP1 KO mice handled a glucose load similarly to WT mice, further confirms the role of RAMP3, and not RAMP1, in glucose control.

To further understand the role of RAMP1 and RAMP3 in influencing spontaneous food intake, detailed meal pattern analyses were performed in chow-fed KO mice. The increase in IMI and meal duration observed in RAMP1 and 1/3 KO male mice, and increased meal size in RAMP1/3 KO males, suggest that these mice are more sated between meals while also displaying delayed or decreased satiation. Decreased meal size is a hallmark of amylin-induced satiation (Lutz et al., 1995; Morley et al., 1997). Thus, the prolonged meals observed in RAMP1 and RAMP1/3 KO mice suggests that amylin acts to terminate meals via the AMY1 receptor. CGRP also acts in the brain to induce satiation (Campos et al., 2016), and binds to AMY1 and AM1, both of which contain RAMP1 (Gingell et al., 2019; Hay et al., 2015). Reduced CGRP signaling in the RAMP1 KO mice could therefore also contribute to the altered spontaneous feeding pattern. Conversely, both male and female RAMP3 KO mice displayed a reduction in IMI, and female RAMP3 KO mice actually consumed less food and smaller meals during the light phase. Such a profile indicates enhanced diurnal satiation in RAMP3 KO mice. One explanation for these paradoxical results is that in absence of RAMP3, the body compensates by over-expressing RAMP1, which would hypothetically enhance the satiating effect of amylin or CGRP, or perhaps RAMP2 whose effect and role are not well studied. Whether global deletion of a single RAMP modifies the expression pattern of other RAMPs has not been investigated.

Because the altered meal patterns in the RAMP-deficient mice likely reflect changes in endogenous amylin and CGRP signaling, we more specifically targeted the involvement of RAMP1 and 3 in amylin signaling by characterizing the responsiveness of WT and RAMP KO mice to exogenous amylin and sCT. WT and RAMP1 KO mice responded in a similar anorectic fashion to treatment with amylin and its receptor agonist, sCT. Amylin failed to reduce food intake in RAMP3 KO mice, while sCT had a similar anorectic effect in WT and RAMP3 KO mice. Meanwhile, RAMP 1/3 KO mice did not respond to the anorectic effect of amylin and were also unresponsive to the anorectic effect of sCT in comparison to WT mice. These data suggest that the RAMP3 receptor subunit is main receptor mediating amylin's acute anorectic response. Indeed, previous studies hypothesized that RAMP3 may be the primary amylin receptor for AP-mediated actions. Earlier work from our group reported CTR+ neurons in the AP preferentially expressed RAMP1 and RAMP3 together, as opposed to either one alone. However, when CTR+ cells only expressed RAMP1 or 3, RAMP3 was the preferred accessory subunit (Barth et al., 2004; Liberini et al., 2016). Further supporting the role of RAMP3 in amylin's anorectic action, a previous study showed that the overexpression of RAMP1 did not alter feeding behavior in chow-fed mice (Zhang et al., 2011), however responsiveness to exogenous amylin was not assessed.

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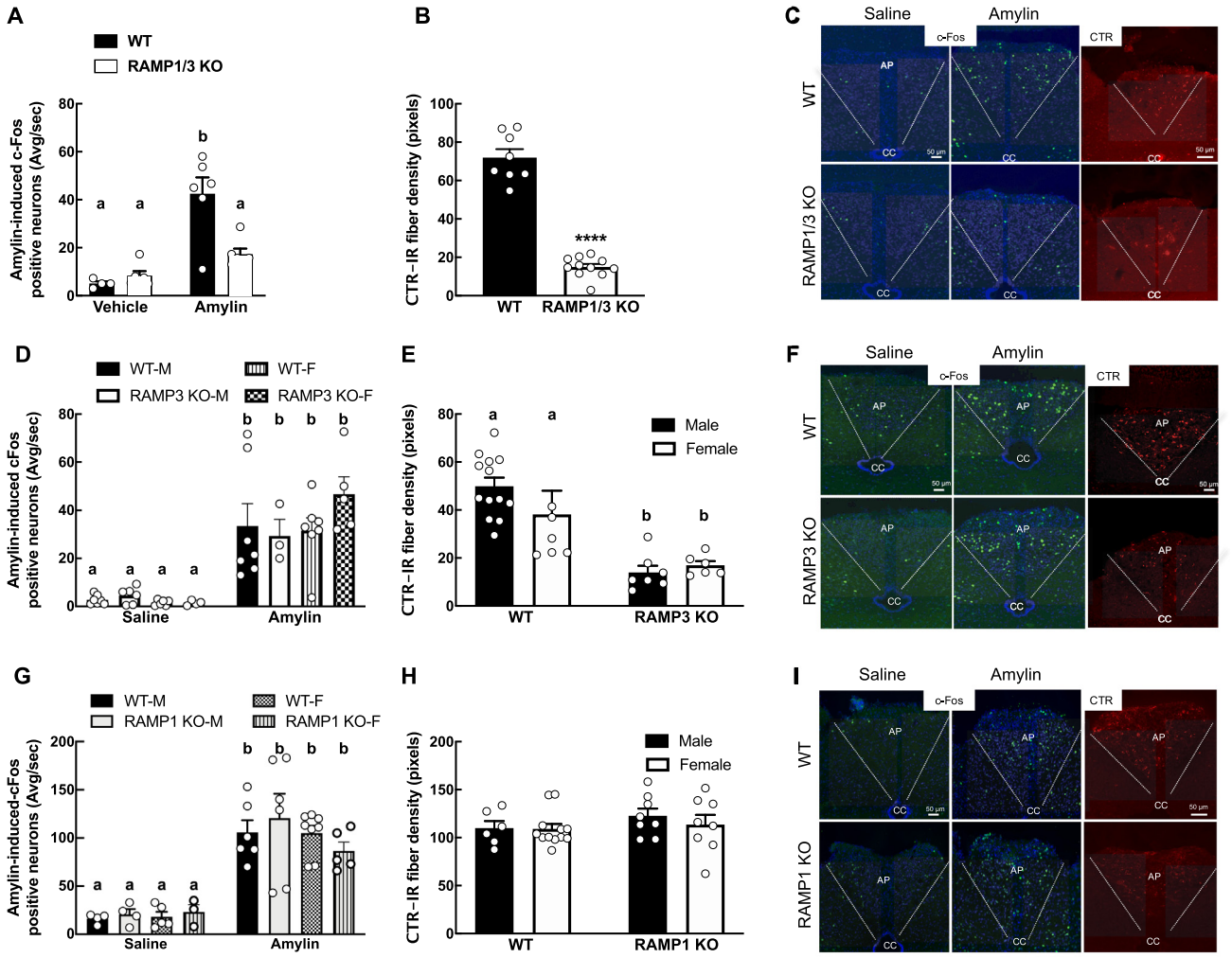

Fig. 6. Amylin-induced c-Fos-positive neurons (**A, D, G**) and CTR density (**B, E, H**) were measured in the AP of WT vs. RAMP1/3 KO male mice (cohort 2–3), RAMP3 KO (cohort 1) and RAMP1 KO (cohort 1) male and female mice 90 minutes after amylin (i.p. 50 μg/kg) or saline injection. (**C, F, I**) 20X representative images of c-Fos and CTR IHC staining; AP brain sections were stained for c-Fos in green and counterstained with DAPI in blue. CTR fibers are stained in red. Values are represented as mean ± SEM. n = 6–7/group for WT/RAMP1/3 KO, n = 8/group for WT/RAMP3 KO, n = 8/group for WT/RAMP1 KO. Statistics: two-way ANOVA with repeated measure followed by post-hoc Sidak's test. Data with differing superscript differ from each other at $P < 0.05$. t-test ****$P < 0.0001$ WT vs KO.

The AP is amylin's and sCT's primary site of action to decrease food intake (Braegger et al., 2014; Lutz et al., 2001), so we sought to determine if RAMP deficiency changes how amylin activates AP neurons. To form a functional amylin receptor both CTR and RAMP need to be present (Hay et al., 2015). However unlike amylin, sCT can bind to CTR in the absence of RAMPs (Hay et al., 2005; Lutz et al., 2000). Amylin-induced c-Fos was blunted in RAMP1/3 KO mice but not in single RAMP3 and RAMP1 KO mice. Thus, it appears that at least one RAMP subunit is needed for amylin to activate AP neurons, as when both are depleted almost no c-Fos signal was observed in AP neurons following treatment with amylin. Interestingly, the CTR fiber density was comparable between WT and RAMP1 KO mice, while it was decreased in RAMP1/3 and single RAMP3 KO mouse models. While this decrease in CTR correlated with the absence of the anorectic effect of amylin in RAMP3 and RAMP1/3 KO mice, only the 1/3 KO mice exhibited a decrease in amylin-induced c-Fos. We can thus hypothesize that a minimum level of CTR is present in RAMP3 KO mice and that this level is sufficient to form AMY1 or AMY2 with the remaining RAMPs and activate c-Fos, but it was not sufficient to produce an anorectic effect. On the contrary, amylin was able to activate c-Fos in the AP and decrease food intake in RAMP1 KO mice, which displayed similar CTR fiber density as WT littermates. This may indicate that both RAMP3 and CTR need to be present for amylin to produce an anorectic effect. Whether the presence of RAMP3 is implicated in CTR transcription or affects CTR transport and integration into the cell membrane remains to be determined. While this report has focused on how RAMP deficiency alters AP responsiveness to amylin, it is important to highlight that RAMPs are located throughout the brain, providing additional routes whereby RAMP deficiency could modify energy balance. Brain nuclei such as ARC, VMN and VTA have been shown to be responsive to amylin, express

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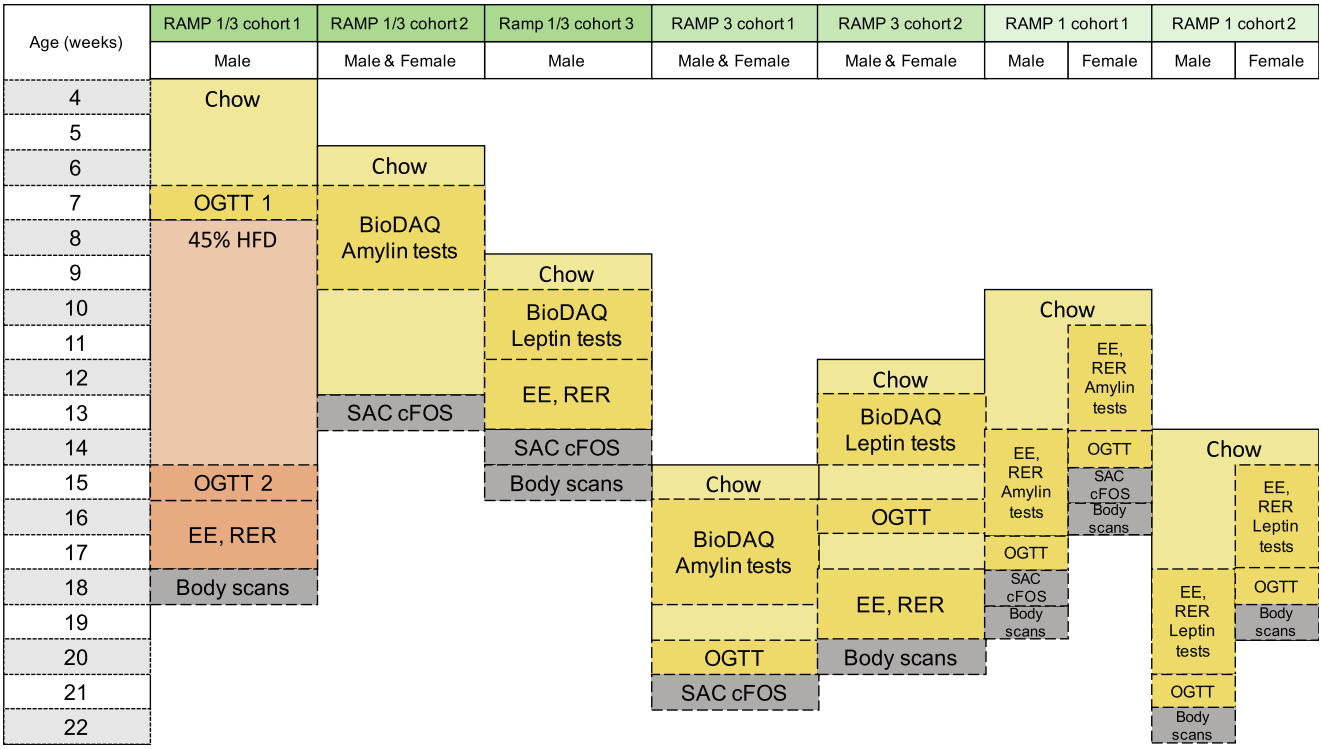

Fig. 7. Flowchart summarizing the experimental procedures and cohorts for RAMP1/3 KO, RAMP3 KO and RAMP1 KO mice.

RAMPs (Le Foll et al., 2015; Mietlicki-Baase et al., 2013), and play a role in the control of food intake (Dunn-Meynell et al., 2016; Mietlicki-Baase et al., 2015; Potes et al., 2010). Notably, we have previously shown that in RAMP1/3 KO mice, amylin-induced pERK was decreased in POMC ARC neurons, and this effect was independent of AP signaling (Lutz et al., 2018).

Our last aim was to determine if RAMP deficiency alters leptin responsiveness. Early investigations into the interaction of amylin and leptin focused on amylin's ability to restore or enhance leptin signaling in the ARC and VMN (Le Foll et al., 2015; Lutz et al., 2018; Roth et al., 2008; Turek et al., 2010). Our more recent work revealed that functional leptin receptors seem to be necessary for amylin signaling in the AP (Duffy et al., 2018), suggesting a bi-directional interdependence of these two hormones. RAMP1/3 KO mice were insensitive to the effect of leptin and leptin + amylin. RAMP1 KO mice demonstrated a tendency toward reduced leptin sensitivity, while RAMP3 KO mice decreased their food intake similarly to WT littermate when injected with leptin + amylin. The consistent, blunted ability of leptin to reduce food intake across RAMP KO models supports the notion that amylin signaling promotes leptin sensitivity, however the present data cannot conclusively decipher whether a specific RAMP is most critical for this effect.

Given the fact that the prevalence of obesity is higher in women (Hales et al., 2018), the interaction between the signaling of estradiol and gut peptides is of great importance. While no sex differences were detected in RAMP1/3 and RAMP3 KO mice, the RAMP1 KO differentially affected male and female mice. Male RAMP1 KO mice exhibited more fat mass, prolonged meals, and were unresponsive to acute amylin effect on food intake, while female RAMP1 KO mice were rarely different from their WT controls. A limited number of studies have investigated the influence of female sex hormones on the expression of RAMPs. Female rats were shown to have significantly lower baseline levels of RAMP1 mRNA in the trigeminal ganglion than males (Stucky et al., 2011) and treatment of estrogen-depleted ovariectomized (OVX) female rats with exogenous estradiol resulted in reduced expression of RAMPs 1, 2, and 3 in uterine tissue (Thota et al., 2003). Whether females also have reduced expression of RAMPs in the brain has not been determined, but it is plausible that the RAMP1 KO affected female mice were less because female mice basally express less RAMP1. An interaction between estradiol and amylin has also been observed. Unlike most satiating hormones, whose effects on food intake are enhanced by estrogens, Trevaskis et al. showed that chronic amylin infusion was more effective at reducing body weight, adiposity and food intake in estrogen-depleted OVX rats than in control female rats or in OVX rats that received estradiol replacement therapy (Trevaskis et al., 2010). Whether these differential affects resulted from differences in RAMP expression was not tested, but it is possible that enhanced amylin, and potentially also CGRP, efficacy resulted from changes in RAMP1 expression in the absence of estrogens.

To conclude, the results from our current study provide evidence for a role of RAMP1 in the mediation of fat utilization, while they suggest a role for RAMP3 in

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glucose homeostasis. One of the RAMP is sufficient for amylin-induced c-Fos in the AP but RAMP3 and CTR need to be both present to produce amylin's anorectic effect. Whether the depletion of RAMPs alters other non-amylin signaling pathways remains to be determined as well.

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CLF, CNB and TAL designed the experiments. BC, SWP, SA, CNB and CLF performed the experiments. CLF, CNB and SWP wrote the manuscript. BC, SWP, SA, CNB, CLF and TAL reviewed this manuscript.

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Declarations of interest: none.

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